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*Effects of arbuscular mycorrhizal symbiosis on  
nitrogen dynamics in Mediterranean agro-ecosystems*

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# 1 INTRODUCTION

Agriculture changes. Thanks to a growing ecological sensibility, international agricultural research is focusing towards sustainable production strategies. These strategies primarily foster to reduce energy input in agro-ecosystems through an optimization of the turnover of the elements and through an improvement of biological control of stresses. So, the sum of this studies leans on the application of the sustainable development in agriculture (Altieri, 1994), which meet to the social expectations of food quality and environmental protection. In sustainable systems, soil microflora and soil fauna are pivotal to control soil borne diseases, to determine species fitness and production quality due to their roles in the biogeochemical cycle of elements. For such matters, scientific research shows growing interest to delve into biodiversity, dynamic and significance of microbial populations in soil and into the mechanisms that rule their biological activity (Werner, 1998; Kennedy, 1998; Bowen & Rovira, 1999).

In the immense soil microbial survey, mycorrhizal fungi and N<sub>2</sub> fixing bacteria (NFB) play a major role as symbionts of cultivated crops. Relating their matter of importance and distribution, NFB-legumes symbiosis has been extensively studied regarding its physiology and benefits to plants (Vance, 2001; Lum & Hirsch, 2003). Mycorrhizas (greek: μύκης (mykes) = fungus; ρίζα (rhiza) = roots, after Frank, 1885) have the same importance and a distribution as widespread as NFB. Mycorrhizas are symbioses between plants (macro- or phyto-biont) and fungi (micro- or myco-bionts). It seems that mycorrhizas appeared on earth about 460 million years ago (Redecker *et al.*, 2000) and helped plants during the early colonization of lands (Pirozynski & Malloch, 1975; Simon *et al.*, 1993; Brundrett, 2002). Thanks to its ancestral occurrence, this association can be found in the 80% of vegetal species (Smith & Read, 1997) and it's responsible of major nutrient and water plant uptake in most of the environments (Harley & Smith, 1983; Trappe, 1987; Allen, 1996), especially in the harshest for plant survival or production (Mosse *et al.*, 1981).

The capability of being infected by mycorrhizas vary with the plant species. Usually legumes are likely to be infected by AM fungi. Some plant species seems to be completely dependent by mycorrhizas for their nutrient uptake and others do not form mycorrhizal associations. The non-mycorrhizal-host traits of some species (manly in the families *Juncaceae*, *Cruciferae*, *Chenopodiaceae*, *Cyperaceae* and *Caryophyllaceae*) (Wang & Qiu, 2006) might be a derived trait and it might be the outcome of specialization regarding, e.g., the plant habitat (Fitter & Moyersoen, 1996; Strack *et al.*, 2003).

## 1.1 CLASSIFICATION AND DISTRIBUTION OF MYCORRHIZAS

Mycorrhizal symbioses can be divided in 7 groups basing on histological (Tab. 1-1) and genetic traits. The most frequent and non-specific ones are between Arbuscular Mycorrhizal Fungi (AMF) or Ectomycorrhizal fungi (ECM) and plants. The others kinds of mycorrhizal symbioses have a certain specificity between some fungi *taxa* and plant families. ECM are distinguished by a dense mycelium sheaths around the roots and particular intercellular hyphal invasion of the plant root cortex forming the so called *Hartig net*. From both of the structures, an intricate hyphal web spreads in the surrounding soil (Bolan, 1991). AMF are polynucleate haploid organisms with an obligate symbiotic status. They are characterized for an active penetration into the root cortex and for the formation of arbuscules into the root cells. Arbuscules are tree stem-like shaped organs which set an extended surface area for nutrient exchanges between the fungus and the cell cytoplasm (Bonfante & Perotto, 1992 and 1995).

Tab. 1-1 - Table summarising key differences between mycorrhizal association types (modified after Smith & Read, 1997).

Type	AMF	ECM	Ectendo-	Arbutoid	Mono-tropoid	Ericoid	Orchid
Septate hyphae	- (+)	±	±	+	+	+	+
Hyphae in cells	+	-	+	+	+	+	+
Hyphal coils	±	-	-	-	-	+	+
Arbuscules	+	-	-	-	-	-	-
Mantle	-	+ (-)	+ (-)	+	+	-	-
Hartig net	-	+	+	+	+	-	-
Vesicles	±	-	-	-	-	-	-
Vegetal taxa symbionts	Vascular plants	Gymnosperms & Angiosperms		Ericales	Mono-tropaceae	Ericales	Orchidaceae
Chlorophyll	+	+	+	±	-	+	±
Fungi	Glomerales	Most Basid-, but some Ascomycetes				Asco-(Basid-)	Basid-

Notes: AMF=Arbuscular Mycorrhizal Fungi; ECM=Ectomycorrhizal Fungi;  
 Ectendo-=Ectoendoid Mycorrhizal Fungi; - = absent; + = present; (+)= sometimes present;  
 (-)= sometimes absent; ± = present or absent; Basid- = Basidiomycetes; Asco- = Ascomycetes.

The genetic organization of AMF consists in several haploid nuclei, up to some hundreds, present within one fungal spore (Viera & Glenn, 1990; Hosny *et al.*, 1998). Questions have been risen about the significance of such nuclei population, about their probable genetic redundancy and the negative effects associated with the lack of sexuality (e.g. slow genetic adaptation and accumulation of deleterious mutations) (Judson & Normack, 1996; Butlin, 2002; Gandolfi *et al.*, 2003). Sanders (2002a and 2002b) hypothesized that nuclei could be differentiated in different groups due to the natural mutations and that mutations are conserved during generations thanks to the

multinucleate nature of the AM cell. In addition, this diversity could have played an evolutionary role (Sanders, 2002a). More genetic differentiation could also have occurred through nuclear drift and anastomoses (Giovannetti *et al.*, 2001) that provided random assortment of nuclei (Bever & Morton, 1999; Sanders, 2002b) among different individuals and through to an uneven distribution of different nuclei from parent to newly formed cells during mitosis (Sanders, 2002a).

Presently, all AMF species are members of the phylum *Glomeromycota* (Fig. 1-1) which seems to have a monophyletic origin. It currently includes 4 orders and 13 families phylogenetically sound for both genetic and morphological traits (Schüßler *et al.*, 2001; Walker *et al.*, 2004; Oehl & Sieverding, 2004). *Glomeromycota* numbers more or less 200 species formally described on the basis of spore morphological traits. Every way, some authors suppose the existence of many unidentified species due to their incapacity or impossibility to sporulate (Bever *et al.*, 1996; Vandenkoornhuyse *et al.*, 2002; Redecker *et al.*, 2003; Wirsal, 2004). The phylogeny of AM fungi is in continuous change. Up to April 2009, 19 genera of AMF can be distinguished (Tab. 1-2). An extensive and up-to-date description of genera and species of AM fungi can be found at the website '<http://www.lrz-muenchen.de/~schuessler/amphylo/>'. It is important to pinpoint that the genus *Glomus* (Fig. 1-1b), subdivided in three clades (Groups Aa and Ab as one single genus and Group B as another one) is not completely characterized at the moment and still considered as *morphotaxon*. The phylogenetic position of family *Entrophosporaceae* is also unclear and not presented in Fig. 1-1b and the genus *Appendicispora* and the family *Appendicisporales* are synonymic to *Ambispora* and *Ambisporaceae*, respectively (Walker, 2008). The family *Gigasporaceae* have been recently split in 4 families: *Gigasporaceae*; *Scutellosporaceae*; *Racocetraceae*; and *Dentiscutataceae*. Finally, it's important to report that the genus *Geosiphon* (with the species mycorrhiza-like fungus *G. pyriformis*) is the only taxon forming symbiosis with cyanobacteria of the species *Nostoc punctiforme* (Kluge *et al.*, 2002). A detailed description of genera can be found in Redecker & Raab (2006) who stated that "environmental studies using phylogenetic methods for molecular identification have recovered an amazing diversity of unknown phylotypes, suggesting considerable cryptic species diversity". Every way, many changes may occur to this classification in the near future, especially for the lower *taxa* for the high genetic variability of AMF, even internal to the species and to the *nuclei* (Sanders *et al.*, 1995; Kuhn *et al.*, 2001; Pawlowska & Taylor, 2004).

AM fungi can be found in several environments (Strack *et al.*, 2003) such as deserts (Corkidi & Rincón, 1997; Dalpé *et al.*, 2000; Titus *et al.*, 2002), rain forests (Brundrett *et al.*, 1999; Guadarrama & Álvarez-Sánchez, 1999; Siqueira & Saggin-Junior, 2001; Zhao *et al.*, 2001; Gaur & Adholeya, 2002), aquatic environments (Khan, 1993), ecosystems with saline (Carvalho *et al.*, 2001; Sengupta & Chaudhuri, 2002) and sodic or gipsic soils (Landwehr *et al.*, 2002). The relatively low number of Arctic and Antarctic environments colonized by AMF is probably due to the lack of inoculum carriers rather than to other causes (Allen, 1996).

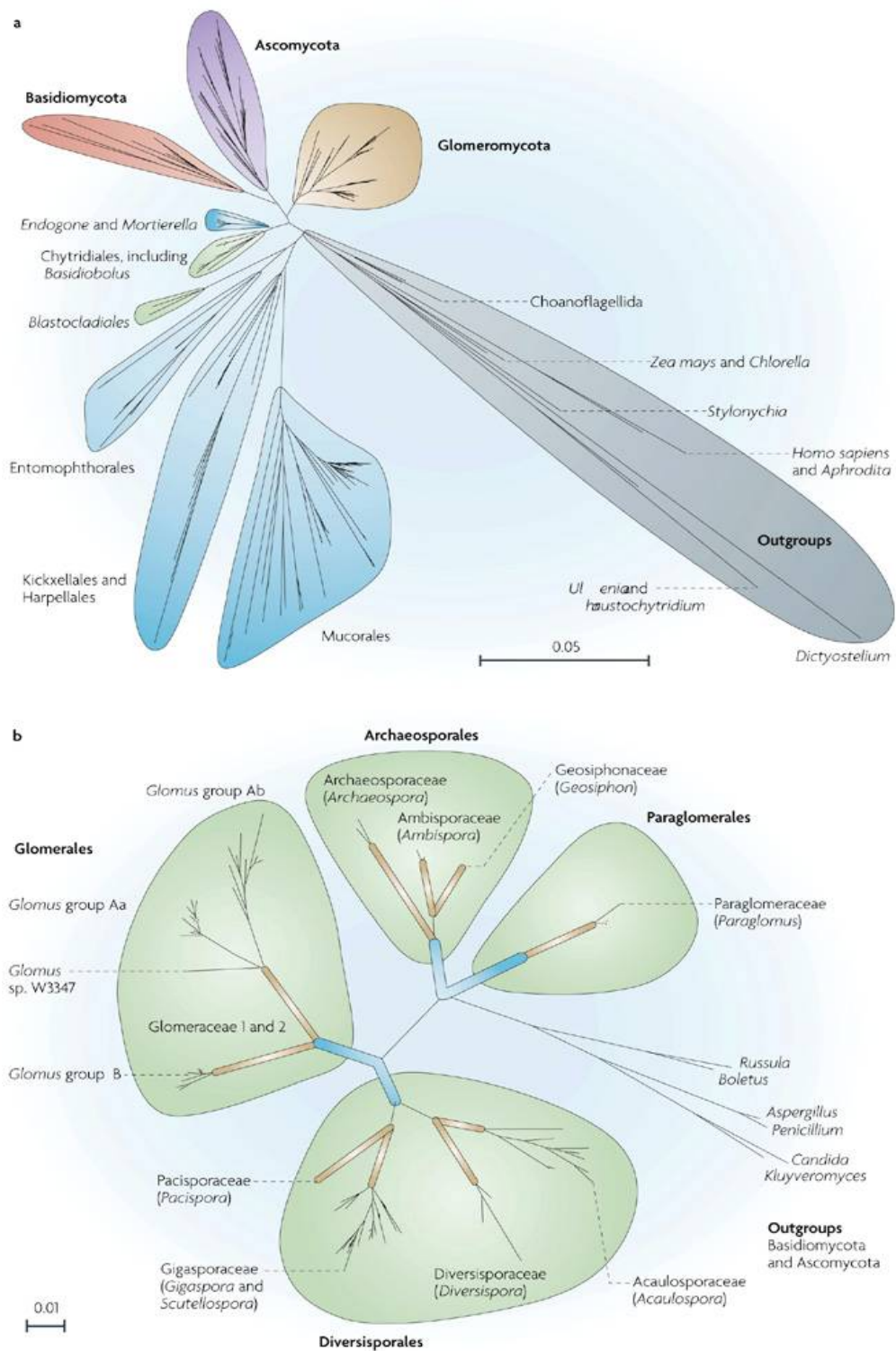


Fig. 1-1 – a) Phylum Glomeromycota positioning in the clade tree of Fungi Reign; b) clade tree of the family of the phylum Glomeromycota (a and b extracted by Schüßler et al., 2001 and modified by Parniske, 2008)

Tab. 1-2 – Classification of AM fungi (extracted by Schübler's database available at <http://www.lrz-muenchen.de/~schuessler/amphylol/>)

Orders (4)	Families (13)	Genera (19)	No. species (214)
<i>Glomerales</i>	<i>Glomeraceae</i>	<i>Glomus</i>	105
<i>Diversisporales</i>	<i>Gigasporaceae</i>	<i>Gigaspora</i>	9
	<i>Scutellosporaceae</i>	<i>Scutellospora</i>	10
	<i>Racocetraceae</i>	<i>Racocetra</i> & <i>Cetraspora</i>	9 & 5
	<i>Dentiscutataceae</i>	<i>Dentiscutata</i> & <i>Fuscutata</i> & <i>Quatunica</i>	7 & 4 & 1
	<i>Acaulosporaceae</i>	<i>Acaulospora</i> & <i>Kuklospora</i>	34 & 2
	<i>Entrophosporaceae</i>	<i>Entrophospora</i> *	2
	<i>Pacisporaceae</i>	<i>Pacispora</i>	7
	<i>Diversisporaceae</i>	<i>Diversispora</i> * & <i>Oliospora</i> *	4 & 1
<i>Paraglomerales</i>	<i>Paraglomeraceae</i>	<i>Paraglomus</i>	3
<i>Archaeosporales</i>	<i>Geosiphonaceae</i>	<i>Geosiphon</i>	1
	<i>Ambisporaceae</i>	<i>Ambispora</i>	8
	<i>Archaeosporaceae</i>	<i>Archaeospora</i> & <i>Intraspora</i>	1 & 1

\* unclear phylogenetic affiliation



## 1.2 GENETIC AND HISTOLOGICAL TRAITS OF THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS

Arbuscular mycorrhizal (AM) fungi are obligate symbionts of plants. Without its host, AM fungi have a relatively short growth (20-30 days) (Bonfante & Perrotto, 1995) after which modifications in fungal morphology stop hyphal growth. It's still unclear why AMF do not grow saprotrophically in absence of a living host, though it has been demonstrated that *Gigaspora margarita* is capable of DNA replication during spore germination and that "possess the machinery needed for DNA replication even in the absence of the host plant" (Bianciotto & Bonfante, 1993; Bonfante & Perrotto, 1995).

When a fungal spore and/or a fungal hypha comes nearby of living host roots, development of mycelium starts with a reciprocal signaling pathway (Paszkowski, 2006). Roots synthesize and release chemicals, mainly flavonoids (Lum & Hirsch, 2003) and strigolactones (Akiyama *et al.*, 2005), leading to intensified elongation and branching of AM hyphae (Tamasloukht *et al.*, 2003). By his own way, as soon as hypha touches the root, it starts forming an *appressorium* and releasing exo- and endoglucanases, cellulases, xyloglucanases and pectolytic enzymes including polygalacturonase (García-Romera *et al.*, 1991; García-Garrido *et al.*, 1992a, 1992b and 1996; Rejon-Palomares *et al.*, 1996), which improve fungal penetration through plant cell walls. The plant facilitates the penetration of the fungus opening an epidermal cleft in the anticlinal cell walls of two adjacent epidermal cells (Parniske, 2004; Demchenko *et al.*, 2004). Then fungal hyphae are allowed to pass intracellularly through an exodermal cell and an adjacent cell from the outermost cortical layer (Hause & Fester, 2005). Genre *et al.* (2005) also showed that *Medicago truncatula* rhizodermal cells form a pre-penetration *apparatus*, a hollow column composed of microtubules, microfilaments and endoplasmic *reticulum cisternae*, that guides the invading hypha through the cell *lumen*. It's also notable that such a *apparatus* is formed before fungal invagination of the plant cell, so before that fungus enters the root cells apoplast. Moreover, the pre-penetration *apparatus* is not induced in plant mutants in which *appressoria* formation is allowed but who are unable to form a complete symbiosis. After having entered the first cell layer, the hyphae pass through the outer layers of living cells, surrounded by a plant perifungal membrane that is histologically continuous with the plasma membrane. This step is maybe the bottleneck of the complete establishment of the AM symbiosis (Parniske, 2004). In *Lotus japonicus* mutant defective for the synthesis of the perifungal membrane, the penetration of the hypha into the outer layers of the plant cell lead to a death of both the plant cell and the hyphal tip (Bonfante *et al.*, 2000).

When the fungus reaches the inner cortex layer, it starts growing and branching throughout the apoplast under the control of the plant (Ivashuta *et al.*, 2005; Paszkowski *et al.*, 2006). During this process, some hyphal branches penetrate inner cortical cells without forming any *appressoria* and, by repeated dichotomous branching, initiate the synthesis of a characteristic tree-like structure, the arbuscule. It is not known what triggers the fungal entrance into the cell (Paszkowski, 2006), maybe a radial sugar gradient between the vascular tissue and the outer cell layers is involved in induction of the arbuscules formation (Blee & Anderson, 1998). Arbuscules surely are the key

organs of the mycorrhiza because are responsible for nutrient exchange between the symbionts. During the arbuscule formation and its life-span, the plant cell undergoes hard morphological and physiological changes. In details, plasmalemma starts to invaginate, the vacuole becomes fragmented and a periarbuscular membrane is synthesized in *continuum* with the plasma membrane (Harrison, 1999), amyloplasts disappear and the number of organelles such as Golgi bodies and plastids increases (Bonfante & Perotto, 1992 and 1995) and form a network covering the arbuscule (Fester *et al.*, 2001). In this network, plastids are connected to each other by tubular structures called ‘stromules’ (Köhler & Hanson, 2000). At the same time, the plant cell *nucleus* increases in size owing to unfolding of its chromatin, though maintaining its ploidy (Berta *et al.*, 1990b), and it moves to a central position, surrounded by plastids forming “octopus-,” “millipede”-like or ring-shaped structures (Strack *et al.*, 2003) whose presence indicates an intensified metabolism. Once the arbuscule and the periarbuscular membrane are completely formed, the plant and the fungus start a reciprocal and active exchange of nutrients. Many transporters and other enzymatic complexes have been identified onto the peri-arbuscular membrane, mainly P transporters (Paszkowski *et al.*, 2002; Harrison *et al.*, 2002; Glassop *et al.*, 2005; Maeda *et al.*, 2006) and ATPases (Gianinazzi-Pearson *et al.*, 1995). In the arbuscules containing cell, particular mycorrhizal induced chitinases are similarly expressed and regulated (Bonanomi *et al.*, 2001). The arbuscule senescens and collapses after some days of activity (4–10 days, Sanders *et al.*, 1977) and the fungal structures are then completely degraded by the plant cell. The plant cell recovers its original morphology (Jacquelinet-Jeanmougin *et al.*, 1987) and can allow a second fungal penetration and arbuscule formation (Alexander *et al.*, 1988 and 1989; Hause & Fester, 2005). Salzer *et al.* (1999) and Walter *et al.* (2000) suggested that arbuscule senescence is somehow dependent by plant responses to intracellular colonization. Moreover, the collapse could be caused by endogenous fungal signaling or coordinated signalling cross-talk (Paszkowski, 2006).

In late phases of the symbiosis, all AM fungi form intra- or inter-cellular vesicles, except for the ones of the genera *Scutellospora* and *Gigaspora*. Vesicles are lipid-rich storage organs (Smith & Read, 1997). Finally, the life cycle of the AM fungi finish and start again with the formation of new extraradical mycelia and spores. Another colonization process is then began by the new spores or by the hyphae in soil (Smith & Read, 1997). The overall extent of AM colonisation is controlled by the plant (Solaiman *et al.*, 2000; Nishimura *et al.*, 2002), but the expression of the related operones is not completely understood (Staehelin *et al.*, 2001). Everyway, the genetic determinants that control the colonization and its efficiency are of great agricultural potential (Parniske, 2004).

### 1.3 DIRECT INTERACTIONS BETWEEN AMF AND BACTERIA

AM fungi can interact with soil bacteria both directly, *via* a trophic and physiological dialogue, and indirectly, by modifying the environment in which bacteria live (Barea *et al.*, 2002; Johansson *et al.*, 2004). In this section, I will focus on direct relations between AM fungi and bacteria. Two kinds of AMF-bacteria interaction can be described: (1) between AM extraradical hyphae and bacteria living in the soil and (2) between AM fungi of the family *Gigasporaceae* and bacteria passing their entire life cycle into the cytoplasm of the AM fungus cells, thus establishing a symbiosis between fungus and bacterium.

Many bacteria live in the rhizosphere and attach to AM hyphae (Bianciotto *et al.*, 1996a). It is not clear if the attachment of some bacterial strain to AM hyphae is governed by a chemical and/or molecular dialogue between AM fungus and bacterium, but it has been demonstrated that some bacterial strains specifically respond to some AM fungi (Andrade *et al.*, 1997; Artursson *et al.*, 2005) suggesting a certain degree of specificity (Artursson & Jansson, 2003; Toljander *et al.*, 2006). Results of both Andrade *et al.* (1997) and Artursson *et al.* (2005) suggest that AM fungi are more commonly associated to Gram-positive and  $\gamma$ -proteobacteria than Gram-negative bacteria. Boddey *et al.* (1991) supposed that mycorrhizal extra radical mycelium (ERM) act as vehicle for spreading of bacteria in soil. The attachment of bacteria to AM hyphae seems to follow a two-step process (Vande Broek & Vanderleyden, 1995). In the first phase, a labile binding, probably an electrostatic attraction, is established between the two kinds of microbes (Artursson *et al.*, 2006). In the second step, cellulose fibrils or other bacterial polymers may be involved in the formation of a more stable binding between AM fungi and bacteria (Bianciotto *et al.*, 2001). The attachment of bacteria on AM hyphae is of great agronomic significance. Indeed, a close contact between bacterial cells and root structures is an important feature for many bacterial effects on plant. Moreover, it is a prerequisite for an effective nodule formation in  $N_2$  fixing symbioses. Regarding AM symbiosis, a close contact of bacterial and fungal cells could be important in determining AM growth and activity. Upon that matter, Azcón (1987) and Linderman (1997) showed that plant growth promoting rhizobacteria (PGPR), a group of microbes whose activity can result in plant benefits, have a stimulatory effect on growth of AM fungi. Moreover, Sanchez *et al.* (2004) found a common gene expression in *M. truncatula* in response to a fluorescent *Pseudomonas* and an AM fungus.

The knowledge about the symbiotic life of some bacteria into AM fungi is still controversial. As already mentioned, some AM fungi from the family *Gigasporaceae* can harbour bacteria living symbiotically into all structures of the AM life cycle, except for arbuscules (Bianciotto *et al.*, 1996b). These authors demonstrated that AM endosymbiotic bacteria are able to complete their whole life cycles within the AM cell and that they are Gram-negative and rod-shaped. AM endosymbiotic bacteria were formerly associated to the genus *Burkholderia*, but recent findings assign it to the species *Candidatus (Ca.) Glomeribacter gigasporarum* (Bianciotto *et al.*, 2003). Different authors attempted to cultivate such bacteria on cell-free media with no results (Bianciotto *et al.*, 2004; Jargeat *et al.*, 2004). However, they found that these bacteria were vertically transmitted by generations of AM fungi through vegetative spores. The

non-cultivability and the relatively little genome size of *Ca. G. gigasporarum* typical of all symbiotic bacteria gives clues about its obligate symbiotic status. Moreover, the coenocytic nature of the AM mycelium surely facilitates the migration of AM endosymbiotic bacteria into the AM fungus (Artursson *et al.*, 2006). This hypothesis is supported by the demonstration of an active bacterial proliferation into the AM fungal mycelium (Bianciotto *et al.*, 2004).

No information is available about the physiological role of AM endocellular bacteria. By comparing genomic library developed from *G. margarita* spores, some authors supposed that some genes of endosymbionts of AM fungi are involved in the phosphate transport, in chemotaxis and in coat formation of bacterial endospores (Ruiz-Lozano & Bonfante, 2000; Minerdi *et al.*, 2002). In particular, Minerdi *et al.* (2001) found a DNA region containing putative *nif* genes in the genome of *Ca. Glomeribacter gigasporarum*, so it could also be possible that these bacteria are involved in the N<sub>2</sub> fixation from atmospheric sources and contribute to N nutrition in the AM fungus. However, no reports are available about the possibility of N<sub>2</sub> fixation in AM fungi.

The process of cyclical infection of AM fungi by bacteria has not been described. However *Geosiphon pyriforme*, a mycorrhiza-like fungus, was found to harbor a cyanobacteria through an endocytotic process (Schüßler & Kluge, 2001) so it is presumable that it also occurs in AM fungi.

### **1.3.1 DIRECT RELATIONS BETWEEN AMF AND RHIZOBIA**

The symbiosis between legumes and AMF displays several similarities to the NFB-legumes symbiosis by a genetic and eco-physiological point of view (Gollotte *et al.*, 2002). Both symbioses share common steps (briefly: attraction, recognition, entrance, establishment, development of a specialized interface and coexistence with host plant) (Lum & Hirsch, 2003). Provorov *et al.* (2002) showed that a lot of mutants lacking for the ability of establishing the N<sub>2</sub> fixing symbiosis (referred as 'Nod-') are also unable to establish the mycorrhizal one (referred as 'myc-'). Despite the obvious differences between the two symbioses and the uniqueness of each of them, many plant genes are expressed with apparent similar functions in both symbiotic relations. These common features induced LaRue & Weeden (1994) and Provorov *et al.* (2002) to suppose that NFB-legume symbiosis evolved from a set of pre-adaptations during co-evolution of plant with AMF.

During early stages of both symbioses, a molecular and chemical dialogue occurs between AM fungi or NFB with each of their respective host by means of flavonoids in plant exudates (Recourt *et al.*, 1992; Harrison & Dixon 1993, 1994) which take part in the recognition of partners and development of associations. In the N<sub>2</sub> fixing symbiosis, the release of plant exudates induces the production of bacterial Nod factors, molecules that dictate the relations between symbionts (Djordjevic *et al.*, 1997). Plant exudates also induce germination and growth of AM hyphae and hyphal branching when the fungus approaches the plant root. These effects are host-specific and occur only at the presence of host exudates as also shown by Elias & Safir (1987), Gianinazzi-Pearson *et al.* (1989) and Giovannetti *et al.* (1993). As already mentioned, plant flavonoids in roots

exudates exert a stimulus on AM infection. Xie *et al.* (1995) showed that the application of exogenous Nod factor also stimulates AM colonization and that this stimulus was correlated with an enhancement of specific plant flavonoids. It has been shown how this stimulus is not only host dependant, but also AMF genus-specific (Chabot *et al.*, 1992).

The attachment and penetration of AMF and NFB into plant roots is a multi-step procedure involving host participation. The first step of AMF infection is the formation of an appressorium. Even if chitin, the major component of fungal tissues, is known to be an elicitor of plant defence reactions, the appressorium contact with epidermal root cell doesn't cause the typical defence reactions from plants, which triggers particular mechanisms in order to allow AMF but no other fungi to enter the root (García-Garrido & Ocampo, 2002). Anyway, AMF entrance into the plant is more dependant by the fungus rather than the plant. With respect to N<sub>2</sub> fixing symbiosis, molecules produced by NFB during the early stages of the infection are related to fungal chitin and it suggests a plant ability to recognize different form of chitins and similar compounds hence regulating its own defenses the penetrations of symbionts. Once entered the roots cells, both AMF and NBF form particular exchange structures, named respectively "arbuscule" and "bacteroid's membrane". Plant always form an interface between root cell and symbiotic structures. The periarbuscular membrane (between plant cell and AMF) carries xyloglucans, nonesterified polygalaturonans, arabinogalactans and hydroxyproline-rich glycoproteins and similar components can be found in bacteroid compartments (Perotto *et al.*, 1990). Finally, the use of Nod- and/or Myc- legume mutants in research has contributed to an enhanced understanding of the signaling process between host plant and micro-symbiont (NFB and/or AMF) (Gollotte *et al.*, 2002), although further studies are needed to a better understanding of the molecular dialogue between symbionts.

#### **1.4 CONTRIBUTION OF AM SYMBIOSIS TO PLANT GROWTH AND NUTRIENT UPTAKE**

AM symbiosis plays a key role in promoting plant growth and nutrient uptake, especially P (van der Heijden & Sanders, 2002; Garg *et al.*, 2006; Hoeksema *et al.*, 2010). Such support mainly occurs in growth-limiting environments (Smith & Read, 1997) or in crops where it isn't possible to use mineral fertilizers (Scullion *et al.*, 1998). Many reports show that AM symbiosis improves growth in drought-stressed conditions (Augé, 2001) and that its effects on drought tolerance can either be mediated by the higher P inflow (Kwapata *et al.*, 1985; Osonubi *et al.*, 1991) or occur irrespective of a higher P uptake in AM than non AM plants (Augé *et al.*, 1994; Schellenbaum *et al.*, 1999). Indeed, AM fungi have side effects on soil hydraulic properties (Augé *et al.*, 2004) and soil structure (Rillig & Mummey, 2006) and these effects help plants to cope with water deficit. In field crops, several reports show that AM fungi inoculation improves plant dry matter (DM) production for economically important *taxa* of plants such as legumes (Fitter, 1985; Barea *et al.*, 1987; Kristek *et al.*, 2005) and grasses (Al-Karaki *et al.*, 2004). Generally AM symbiosis enhance root biomass thanks to the improved mineral nutrition, but sometimes it induced depressive effects on root DM (Koide, 1985; Fitter, 1977) and root length (Berta *et al.*, 1990a). The AM symbiosis influences many aspects of root physiology such as plant rooting and root morphology,

nutrient acquisition rates and root reactions against stresses (Atkinson *et al.*, 1994; Kapulnik & Douds, 2000; Berta *et al.*, 2002). It has been sometimes reported that AM may have a negative effect on plant growth (Ryan & Angus, 2003; Li *et al.*, 2008) but it has been found only for some plant species grown in pot in symbiosis with single AM strains.

The effects of the AM symbiosis on nutrient uptake are clearly visible when nutrient availability for plants is scarce due to low nutrient concentration in soil or to low nutrient diffusibility (George, 2000). It is the case of nutrients such as P, Zn and Cu and others minerals (Barea *et al.*, 2005), though AM symbiosis is also efficient in enhancing uptake of N (Jin *et al.*, 2005; Govindarajulu *et al.*, 2005). The enhanced nutrient uptake by AM plants in comparison to non-AM plants mainly depends on a better scavenging of soil area thanks to (1) the little dimensions of AM hyphae, which can access nutrient sources normally unavailable to plant root and root hairs (Chen *et al.*, 2005; Jakobsen *et al.*, 2005); and to (2) the possibility for AM fungi to absorb nutrients in forms unavailable for plants, such as organic N or organic P (Jayachandran *et al.*, 1992; Tarafdar & Marschner, 1994; Koide & Kabir, 2000; Hodge *et al.* 2001). It has been shown that AM hyphae have an average diameter of 3 to 7  $\mu\text{m}$  while the diameter of the finest root hair is 5 to 20  $\mu\text{m}$  (Wulfsohn & Nyengaard, 1999; Bago, 2000; Dodd *et al.*, 2000) AM hyphal length densities may be up to hundreds-fold greater than root length densities. This features allow AM fungi to better explore the soil volume and so raise the probability that ions with low diffusion coefficient are absorbed (Miller *et al.*, 1995). Moreover, AM hyphae are more effective than roots to absorb and utilize nutrients (O'Keefe & Sylvia, 1992; Cui & Caldwell, 1996).

The area of soil in which AM symbiosis spread and establish its activity it's also called mycorrhizosphere (Barea *et al.*, 2002). In this area AM influences bacterial activity (Azcón-Aguilar & Barea, 1992; Bianciotto *et al.*, 2001; Bianciotto & Bonfante, 2002) and bacterial populations (Mansfeld-Giese *et al.*, 2002; Marschner & Timonen, 2005); it contributes to the formation of water-stable aggregates (Miller & Jastrow, 2000; Jeffries *et al.*, 2002); it contributes to the reduction of stresses by pollutants (Gianinazzi *et al.*, 2002; Gianinazzi e Schüepp, 1994; Bethlenfalvay e Linderman, 1992) and it plays a role in the defense of plants against biotic stresses (Rabie, 1998; Werner *et al.*, 2002). The sum of these effects is important in sustaining plant growth. The AM contribution on the suppressed plant yield loss due to diseases are related to a number of suggested mechanisms as: improved nutrition (Declerck *et al.*, 2002), competition for colonization sites (Fitter & Garbaye, 1994), production of AM anti-microbial compounds (Benhamou *et al.*, 1994), and priming of plant immune system (Pozo *et al.*, 2002; Pozo & Azcón-Aguilar, 2007).

In order to perform its activities into the mycorrhizosphere and plant roots, the AM symbiosis has a very high carbon demand and act as an important carbon sink for the plant: AM fungi can drain up to the 20% of the total carbon fixed by the plant (Bago *et al.*, 2000; Johnson *et al.*, 2002), irrespective of plant P or N status (Wright *et al.*, 1998a and 1998b). If we take into account that AM plants often have a higher DM production than non-AM plants, it means but that the improvement of plant photosynthesis by AMF is higher than the AM carbon demand, as results by many authors suggest (Harris *et al.*, 1985; Ames e Bethlenfalvay; 1987). Plant regulate the amount of carbon transferred to the AM fungus. This regulation relies on the net

transfer of P, and probably also N, by the AM fungus to the plant (Fitter, 2006; Javot *et al.*, 2007). Sometimes AM symbiosis can even suppress plant P transporters that are expressed under P deficient conditions, as demonstrated with molecular tools by Liu *et al.* (1998) and Burleigh & Harrison (1999). This increases plant reliance on AM symbiosis for P uptake so improving the transfer of C from plant to fungus and could be an important issue for the survival of the AM fungi.

#### **1.4.1 EFFECTS ON P UPTAKE**

Phosphorus is the most limiting element after N for plant growth and production (Vance *et al.*, 2000) and over 80% of P in soil is in unavailable forms (organic or insoluble) to plants (Holford, 1997; Schachtman *et al.*, 1988). Inorganic P, such as other nutrients, has a very low soil diffusibility and the high plant demand and uptake frequently generate a P depletion zone around of the root surface (Trenbath, 1976; Jungk & Claassen, 1989). In order to face its needs, plant have evolved several strategies to obtain P when it is not available (Vance *et al.* 2003; Hammond *et al.* 2004; Raghothama, 2005) among which AM symbiosis is one of the most important (Lum & Hirsch, 2003).

The importance of AM symbiosis to absorb P from soil is related to the ability of AM hyphae to scavenge soil for P reserves normally unavailable to plants (Miyasaka & Habte, 2001) thanks to four features: (1) AM extraradical mycelium (ERM) is able to explore a higher soil volume than plant roots; (2) AM hyphae have a very small diameter if compared to both plant root and plant root-hair, thus increasing P absorbing surface; (3) AM fungi form polyphosphates in its cells, thus lowering internal P concentration and generating a high P gradient in AM cells respect to soil matrix; and (4) AM fungi produce organic acids and phosphatases which catalyze the release of P from organic complexes (Bucking & Shachar-Hill, 2005). Mycorrhizal P uptake pathway is regulated by molecular mechanisms which imply the expression of mycorrhizal P transporters to load P from soil into AM hiphae (Harrison & van Buuren, 1995), its active translocation into the intraradical mycelium and delivery to the host root by means of specific P transporters (Paszkowski *et al.* 2002; Glassop *et al.* 2005).

Reports on higher plant P uptake of AM plants than non mycorrhizal plants are too much to be all reported (McGonigle & Fitter, 1988; Crush, 1995; Ning & Cumming, 2001; Cavagnaro *et al.*, 2003; Gazey *et al.*, 2004; van der Heijden *et al.*, 2006; Schweiger *et al.*, 2007). The most of pot studies involving AM fungi shows a higher P uptake, even if it not always related to plant growth responses (Smith *et al.*, 2003, 2004). Koide & Kabir (2000) showed that, in monoxenic conditions, mycorrhizal ERM was able to access to an organic P sources and transfer it to plant. On the other side, in non-mycorrhizal roots, P availability for plant is exclusively related to the presence and length of the root hairs (Marschner & Dell, 1994). The efficiency of P absorption by AM fungi varies with the AM fungus species (Jakobsen *et al.*, 1992). In field conditions, AM fungi are normally present and studies about their importance in plant P uptake are not easy to perform because of the problems encountered in suppressing natural AM infection (Pedersen & Sylvia, 1997). However, many authors have attempted to perform field experiments, mainly focusing on the benefits of an enhanced

AM infection, and results seem interesting. Schweiger & Jakobsen (1998) directly measured P uptake by AM symbiosis in field grown wheat and found that the symbiosis is responsible for the most of P absorbed by plants thus confirming the results by Liu *et al.* (1998) and Burleigh & Harrison (1999). Black & Tinker (1977) found a higher total P uptake in mycorrhizal potatoes than non-mycorrhizal control, due to the higher DM yield because no differences on P concentration due to AM symbiosis were observed. Rangeley *et al.* (1982) had similar result in field grown white clover, while Hayman & Mosse (1979) found both a higher P concentration and higher total DM yield in AM clover than non AM control, above all in low-P fertilized soil. In pea, Kristek *et al.* (2005) found higher P concentration and P yield in AM than non-AM plants, especially in drought-stressed conditions. Finally, Al-Karaki *et al.* (2004) found that AM inoculation was capable of increasing P concentration and uptake in field grown wheat both in well-watered and drought-stressed conditions. It has sometimes been reported a null effect of AM symbiosis on P uptake in field grown soybean (Ganry, 1982), but it probably occurred because of the low soil P concentration (4.2 ppm measured with the Truog method) that impaired plant growth.

#### **1.4.2 EFFECTS ON N UPTAKE**

AM symbiosis could take up N from soil (Miller & Cramer, 2004) and further translocate it to the plant (Chalot *et al.*, 2006). It has been shown that AM fungi respond to different N sources or N availability (Breuninger *et al.*, 2004; Cappellazzo *et al.*, 2007). However, few studies are available on AM effect on plant N status and the impact of N absorbed by AMF on plant N balance is still unclear. AM symbiosis is active in the absorption of N from inorganic sources (Ames *et al.*, 1983; Johansen *et al.*, 1993). In pot, using  $^{15}\text{N}$ -labelled nitrate as fertilizer, Tobar *et al.* (1994) shown that AM symbiosis considerably contributed to N uptake by lettuce both in drought-stressed and in well-watered conditions. The authors concluded that AM symbiosis is active in the uptake of nitrate from soil. However, AM symbiosis is better suited to take up N when it is in  $\text{N-NH}_4^+$  form as shown by Hawkins *et al.* (2000).

Recent findings shows that AM fungi possess the machinery for a direct N uptake from organic sources (Cappellazzo *et al.*, 2008). After an experiment in monoxenic conditions, Hawkins *et al.* (2000) showed that the presence of the AM fungus was able to absorb N from organic sources and transfer it to the host plant. They also showed that the amount of N adsorbed by the AM fungus from organic sources depends on the organic N form and on the AM fungus strain. The results from Hawkins *et al.* (2000) confirm the ones achieved by Cliquet *et al.* (1997) who showed that presence of AM symbiosis is responsible for an enhanced N acquisition from soil as both nitrate and amino-acids in ryegrass grown in a microcosm system. Such results accord with early and recent evidences about the ability of AM fungi to promote organic nitrogen decomposition and its further uptake (Hodge *et al.*, 2001; Whiteside *et al.*, 2009), but these effects have been studied only in microcosm systems and are not confirmed by pot and field studies. In addition, the mechanisms implied in the AM stimulus of organic N mineralization are still uncertain. In particular it is not clear if AM fungi promote the direct uptake of organic nitrogen, or if they stimulate the activity of N-mineralizer bacteria and promote the uptake of mineralized N.



Many legumes access to the atmospheric N source thanks the N<sub>2</sub> fixing symbiosis and release great amount of N in soil as both dead tissues and exudates. When a legume is intercropped with a non N<sub>2</sub> fixing species, N derived from the atmosphere (Ndfa) can become available to the non-fixing plants *via* a N transfer (Simard *et al.*, 2002; Høgh-Jensen, 2006). The AM symbiosis play an important role in the phenomenon of the N transfer between companion crops. First, AM fungi can mediate direct N transfer between a N donor and a N receiver plant (He *et al.*, 2003) by directly linking the root systems of companion crops. Secondly, AM fungi can absorb root N rich exudates and further transfer it to the host plants (Kapulnik & Douds, 2000; Paynel *et al.*, 2001). The amount of N transferred by AM hyphae is variable. Results from pot experiments suggest that AM symbiosis may facilitate interplant N transfer between legumes and grasses (Hamel *et al.*, 1991; Frey & Schüepp, 1992 and 1993), while others obtained no AM effect on interplant N transfer (Rogers *et al.*, 2001). In field, very few reports are available about AM effects on N transfer and results are still inconclusive.

#### **1.4.2.1 Effects on symbiotic N<sub>2</sub> fixation**

Symbiotic N<sub>2</sub> fixation is fundamental in the biogeochemical cycle of N. It represents the major N input for legumes in almost all environments and it is responsible for the most of available N to all plant communities. AM symbiosis can enhance legume symbiotic N<sub>2</sub> fixation (Barea *et al.*, 2005; Antunes *et al.*, 2006a; Chalk *et al.*, 2006) and receive in turn N as some report seem to suggest (Scheublin & van der Heijden, 2006).

The interactions between AMF- and NFB-legumes symbioses can occur at both colonization and nutritional level. The nutritional aspect is obviously related to the enhanced nutrition of P, Zn, Cu and other immobile nutrients supplied by the AM fungi and important for the NFB-legumes symbiosis (Clark & Zeto, 2000; Vance, 2001). Li *et al.* (1991) found that Cu concentration and quantity in *Trifolium repens* was enhanced by AM symbiosis due to AM hyphal uptake. As a matter of fact, P is fundamental for symbiotic N<sub>2</sub> fixation. In non limiting conditions, legume nodules have a 2-3 fold P-dependency than roots in which are formed (Mosse, 1986; Almeida *et al.*, 2000; O'Hara, 2001; Cuttle *et al.*, 2003), especially in the earlier stage of their development (Smith *et al.*, 1979; Asimi *et al.*, 1980; Drevon e Hartwig 1997; Tang *et al.*, 2001). P deficiency reduces the number and mass of nodules and the activity of N<sub>2</sub>-ase; it increases oxygen permeability of bacteroids so causing an oxidative damage to the N<sub>2</sub> fixing enzymatic machinery (Hunt & Layzell, 1993; Ribet & Drevon 1995; Vadez Rodier *et al.*, 1996). AM symbiosis can also directly affect nodules or bacteroids thus establishing a tripartite symbiosis between AM fungi, N<sub>2</sub> fixing bacteria and legumes. It is not known how the AM symbiosis directly affects the NFB-legume symbiosis. However it has been shown that AM fungi reduce the oxidative damage to nodules (Ruiz-Lozano *et al.*, 2001a) and plant (Goicoechea *et al.*, 1998; Porcel *et al.*, 2003).

AM symbiosis seems to cooperate with NFB-legume symbiosis also in alleviating water or salinity stress for the host plant (Azcón *et al.*, 1988; Azcón & El-Atrash, 1997). The stimulus of AM symbiosis to N<sub>2</sub> fixation and to legume growth is dependant on the combination of rhizobial strain and AM fungus (Rao *et al.*, 1986). In a *ad hoc*

experiment, Azcón *et al* (1991) showed that inoculation of alfalfa grown in pot with different AM fungi and rhizobial strains resulted in different plant growth, %Ndfa (the percentage of total Ndfa above total plant N content) and N and P uptake. Similar results were found by Xavier & Germida (2002), who showed that lentil response (in terms of growth, harvest index, N and P content) to inoculation with different AM fungi or *R. leguminosarum* strains varied both with the *Rhizobium* strain and with the AM fungus. In field conditions, very reports are available on the effects of AM fungi on N<sub>2</sub> fixation by legumes. In soybean, Ganry *et al.* (1985) achieved higher Ndfa in AM than non AM crop when it was grown with a soluble phosphate fertilizer in a low P containing soil, but the same authors reported that AM inoculation didn't produce any growth or Ndfa benefit when no P fertilizer was added.

The tripartite symbiosis has a very high carbon cost, as also shown by several authors (Pang & Paul, 1980; Kucey & Paul, 1982; Harris *et al.*, 1985). Generally, legumes inoculated with effective strains of AM fungi and N<sub>2</sub> fixing bacteria have a higher photosynthesis rate than non-infected plants and this compensate for the C cost of the microsymbionts. In forage legumes subjected to mowing, photosynthesis rates are reduced and the AM symbiosis could compete with the NFB for carbon in the early regrowth stage. The response of both symbioses to above-ground plant removal is similar, but AM symbiosis seems to suffer less than nodules after plant clipping: nodules lose weight more rapidly than roots (Bayne *et al.*, 1984), especially during the first 15 days after clipping (Vance *et al.*, 1979), while AM fungal biomass is reduced less than root biomass (Bayne *et al.*, 1984; Allsopp, 1998) and AM root colonization can even increase (Eom *et al.*, 2001; Pietikäinen *et al.*, 2009). In this phase, the competition for carbon between AM fungi and NFB can negatively affect the N<sub>2</sub> fixation rates of the N<sub>2</sub> fixing symbiosis. Using field-grown *Hedysarum coronarium* subjected to repeated harvests, Barea *et al.* (1987) obtained a higher amount of Ndfa in the AM inoculated than the not inoculated crop and stated that a effective AM fungi-legume combination could improve legume growth, N uptake and Ndfa as just as a P fertilization. In a similar experiment, Shivaram *et al.* (1988) found a higher amount of Ndfa in the AM than non-AM inoculated *Macroptilium atropurpureum*. However both authors (Barea *et al.*, 1987; Shivaram *et al.*, 1988) found no effects of AM inoculation on the %Ndfa indicating that the higher amount of Ndfa in AM inoculated than not inoculated crops depended solely on the promotion of plant growth and not by a stimulus of the N<sub>2</sub> fixation activity.

## 1.5 EFFECTS OF AM SYMBIOSIS ON SOIL BACTERIAL ACTIVITY AND COMMUNITIES

The net of AM extraradical hyphae represent an ecological niche for bacteria. Bacteria living in the mycorrhizosphere are affected by AM fungal activity both directly, by its effects on soil matrix, and by indirectly influencing plant physiology and its relative effects on rhizosphere. Mycorrhizas also affect the community composition of soil bacteria (Marschner *et al.*, 2001; Artursson *et al.*, 2005) by different mechanisms: (1) modifying mycorrhizosphere pH (Bago & Azcón-Aguilar, 1997), (2) affecting activity of free enzymes (Tarafdar & Marschner, 1994), (3) competing for inorganic nutrients (Christensen & Jakobsen, 1993), (4) decreasing the amount of root

exudation (Graham *et al.*, 1981; Dixon *et al.*, 1989) and (5) modifying root exudates compositions (Po & Cumming, 1997; Marschner *et al.*, 1997). In addition, AM fungi release exudates which either promote or impair bacterial development and activity (Toljander *et al.*, 2007). One of the most important AM exudates is surely glomalin (Rillig *et al.*, 2002), a glycoprotein whose concentration is related to water stability of soil aggregates (Wright & Upadhyaya, 1998). The net of AM extraradical hyphae itself contribute to soil aggregates stability by enwrapping soil particles (Rillig & Mummey, 2006). Because microorganisms are mainly present in soil aggregates, AM activity in soil can have positive effects on soil microbial population (Andrade *et al.*, 1998). Moreover, it seems that bacteria can use senescent structures of AM fungi as *pabulum*, as also suggested by the enhanced chitinolytic activity in rhizosphere of AM plants (Abdel-Fatah & Mohamedin, 2000).

Usually AM fungi decrease the growth rate of bacteria (Christensen & Jacobsen, 1993) or induce them in a state of starvation (Marschner & Crowley, 1996; Marschner *et al.*, 1997), though AM fungi may induce an increase in certain functional group of bacteria as actinomycetes and phosphate-solubilizing bacteria (Secilia & Bagyaraj 1987; Kothari *et al.*, 1991; Posta *et al.*, 1994; Toro *et al.*, 1997). With respect to N cycle, Amora-Lazcano *et al.* (1998) found that AM symbiosis increased the population density of ammonia oxidizers, while reducing those of ammonifier and nitrifier bacteria.

## 1.6 AIMS OF THE WORK

The present thesis aimed to obtain information about the contribution of the AM symbiosis in alleviating the effect of abiotic stresses on crops grown in a typical Mediterranean environment and to test if AM symbiosis affects symbiotic N<sub>2</sub> fixation and organic N mineralization activities and finally plant N uptake.

In particular, two experiments were carried out. The first trial was performed under field conditions and had the specific aim to determine the effect of AM symbiosis on forage yield, quality, and biological N<sub>2</sub> fixation of berseem (*Trifolium alexandrinum* L.) grown under both well-watered and drought-stressed conditions.

The second trial was performed in pots to test the hypothesis that plant N uptake would be increased by the direct or indirect effects of AM fungi on OM decomposition process. In order to achieve this objective, it has been studied the effect of plant inoculation with AM fungi on soil enzymatic activity and microbial community, as well as the effects of AM symbiosis on plant N capture from different sources of organic matter.

## 2 MATERIALS AND METHODS

### 2.1 EXPERIMENT 1

Several pot studies have shown the beneficial effects of AM symbiosis on plants grown in water-stressed conditions; even if in other research the effects were absent or negative (Augé, 2001). However, limited information is available on the effects of AM symbiosis on crop drought tolerance under field conditions. In many Mediterranean areas, spring rainfall is scarce, which stresses plants and limits crop productivity. In such environments, AM symbiosis could play an important role in alleviating the effects of drought on crop yield and quality. The aim of this field experiment was to determine the effect of AM symbiosis on growth of berseem (*Trifolium alexandrinum* L.) grown under both well-watered and drought-stressed conditions. This experiment was funded by the *progetto SI.CO.BIO.S*, Regione Siciliana, Italia.

#### 2.1.1 SITE DESCRIPTION

The experiment was carried out in 2007–2008 at the Pietranera field station, in Sicily (Italy) in a Mediterranean area (37°33'N – 13°30'E, 170 m a.s.l.) on a Vertic Haploxerept soil on which wheat was previously grown. The topsoil (0–40 cm) characteristics were: 38% clay, 25% silt, and 37% sand; pH 8.4; 1.27% organic matter; and 0.85% total N. The experimental site has a semi-arid climate (as defined by Emberger, 1955). Long-term mean annual rainfall is ~550 mm, mostly during the autumn–winter period (74%), and with a lesser amount during spring (18%). The mean minimum and maximum temperatures are 10.0 °C and 23.4 °C, respectively. Natural AM spore population in the native field involved the genera *Glomus* and *Acaulospora*. Overall AM spore density was 5 spores 10 g<sup>-1</sup> air-dried soil. AM hyphal mass was not detected.

#### 2.1.2 TREATMENT ESTABLISHMENT AND CROP MANAGEMENT

There were two treatments: (1) soil moisture regime: rainfed (DS) or well-watered (WW); (2) crop mycorrhization: AM inoculation (+AM) or AM suppression (–AM). Minimal rainfall occurred during the spring (55% below long-term average), resulting in drought-stressed conditions for crops in the rainfed treatments. So, well-watered crops received sprinkle irrigation (20 mm at 116 days after sowing [DAS] and 70 mm at 123 DAS). Suppression of mycorrhizal symbiosis was achieved by spraying plots with systemic fungicides as a drench. In order to suppress spore germination, *Captan* (20 mg a.i. m<sup>-2</sup>), *Carbendazim* (20 mg a.i. m<sup>-2</sup>), and *Benomyl* (20 mg a.i. m<sup>-2</sup>) were used at sowing. During the growth of the crops, *Benomyl*, *Fenpropimorph*, and *Carbendazim* were applied once per month at a rate of 10, 5, and 5 mg a.i. m<sup>-2</sup>, respectively in order to reduce AM hyphal growth and AM symbiotic activity. These fungicides are capable

of suppressing AM symbiosis without affecting plant growth (Dodd & Jeffries, 1989; Sukarno *et al.*, 1993; Udaiyan *et al.*, 1995; Kjølner & Rosendahl, 2000; Schweiger *et al.*, 2001). Mycorrhizal sub plots received an equal amount of water. AM inoculation involved the application of a commercial AM inoculum at a rate of 12 g per kg of seed following the manufacturer's recommendations. The inoculum consisted in pure spores of *Glomus intraradices* Schenk and Smith (1000 spores g<sup>-1</sup>) and *Glomus mosseae* (T.H. Nicolson and Gerd.) Gerd. and Trappe (1000 spores g<sup>-1</sup>). The experimental design was a split plot design replicated 4 times with water regime as the main plot and mycorrhization as the sub-plot. Sub plot was 72 m<sup>2</sup>. The experimental design included all factorial combinations.

Soil was ploughed at 30 cm depth on summer and arrowed on autumn to control weeds and prepare suitable seedbed conditions. Such tillage practices presumably reduced the amount of natural AM inoculum, especially the AM hyphal mass (Kabir, 2005). Seeds (2.85 g 1000 seed<sup>-1</sup>) of berseem (*T. alexandrinum* cv Lilibeo) were sown by hand at the 3<sup>rd</sup> of January 2008 in rows 25 cm apart. Sowing density was 1200 viable seeds m<sup>-2</sup>. Weeds were removed by hand.

Plots were cut at 5 cm stubble height at 76 (first cut) and 116 (second cut) DAS. After the second cut, berseem regrowth was measured cutting different microplots (0.6 m<sup>2</sup> wide) at 7, 14, 21, and 28 days after the second cut (DAC).

The <sup>15</sup>N isotope dilution technique was used to estimate N<sub>2</sub> fixation by berseem, using annual ryegrass (*Lolium multiflorum* var. *westerwoldicum* cv. Elunaria) was used as the reference crop (ryegrass received the same treatments as berseem; sowing density was 1200 viable seeds m<sup>-2</sup>). The <sup>15</sup>N-labelled fertilizer ([NH<sub>4</sub>]<sub>2</sub>SO<sub>4</sub> with an isotopic composition of 10 atom% <sup>15</sup>N) was applied at 116 DAS (second cut) following the application procedure described by Høgh-Jensen & Schjørring (1994). Briefly, <sup>15</sup>N-labelled fertilizer was applied as drench at a rate of 8 kg N ha<sup>-1</sup> to two microplots in the middle of each sub plot. The area of <sup>15</sup>N-fertilized microplots was 2.25 m<sup>2</sup>. The rest of the plot outside this area received an equal amount of non-labelled ammonium sulfate.

### 2.1.3 PLANT HARVEST AND ANALYSIS

Epigeic and hypogeic sample areas of berseem and ryegrass were harvested and analyzed for root AM infection and N concentration as shown in Tab. 2-1.

*Tab. 2-1 – Table summarizing date of sampling and analysis on berseem (B) and ryegrass (R). DAS and DAC for ‘days after sowing’ and ‘days after the second cut’, respectively.*

<i>DAS</i>	<i>DAC</i>	<i>Description</i>	<i>% AM colonization</i>	<i>Biomass</i>	<i>N and <sup>15</sup>N concentration</i>
55	-		<i>B+R</i>		
76	-	<i>first cut</i>		<i>B+R*</i>	
116	0	<i>second cut</i>		<i>B+R*</i>	
123	7	<i>regrowth</i>		<i>B</i>	
130	14	<i>regrowth</i>	<i>B+R</i>	<i>B+R</i>	<i>B+R</i>
137	21	<i>regrowth</i>		<i>B</i>	
144	28	<i>regrowth</i>	<i>B+R</i>	<i>B+R</i>	<i>B+R</i>

*\* Ryegrass was harvested above cutting height, only.*

At each cut, the sample areas were harvested both above and below ground by removing the top 20 cm of soil. Plants were counted and separated into taproots, removed leaves, and stems and heads. At first cut (76 DAS) and second cut (116 DAS), residual leaves and stems (below cutting height) were also separated. The fresh weight of each sample was determined, and the leaf area of the leaves (separately for residual and removed at the first and second cut) was immediately measured on a 10 g subsample. Each subsample was oven dried and weighed. Similar measurements were done for ryegrass.

Biomass samples of both berseem and ryegrass, collected at 14 and 28 DAC, were analyzed for total N and <sup>15</sup>N enrichment ( $\delta^{15}\text{N}$ ), using an elemental analyzer – isotope ratio mass spectrometry (EA-IRMS, Carlo Erba NA1500).

At 7, 14, 21, and 28 DAC, ~1 kg soil was taken from two soil layers (0-20 cm and 20-40 cm depth), weighted and dried at 105° until constant weight. Soil dry weights were recorded and soil moisture content was measured by gravimetric method.

At 55 DAS and at 14 and 28 DAC, five plants with their roots were collected in each treatment by careful excavation the top 20 cm of soil and taken to laboratory.

Lateral roots were excised (root tips were discarded), rinsed free of soil and washed in distilled water, cut in pieces of 1 cm length and mixed. A representative sample of roots (more or less 2 g) per sample area was cleared with 10% (w/v) KOH and stained with 0.05% (v/v) trypan blue using the method described by Phillips & Hayman (1970). Root colonization by AM fungi was further measured according to Giovannetti & Mossee (1980).

#### **2.1.4 CALCULATIONS AND STATISTICAL ANALISYS**

Data on  $^{15}\text{N}$  enrichment of biomass were used to calculate the percentage of clover nitrogen derived from symbiotic  $\text{N}_2$  fixation (%Ndfa) according to Fried and Middleboe (1977):

$$\%Ndfa = \left(1 - \frac{\text{Atom}\%^{15}\text{N}_{\text{berseem}}}{\text{Atom}\%^{15}\text{N}_{\text{ryegrass}}}\right) \times 100$$

where  $\text{Atom}\%^{15}\text{N}_{\text{berseem}}$  represents the  $\text{Atom}\%^{15}\text{N}$  excess of berseem tissue, and  $\text{Atom}\%^{15}\text{N}_{\text{ryegrass}}$  represents  $\text{Atom}\%^{15}\text{N}$  excess of ryegrass tissue. The  $^{15}\text{N}$ -natural abundance of the atmosphere (0.3663%  $^{15}\text{N}$ ) was used for calculating the  $\text{Atom}\%^{15}\text{N}$  excess of both crops. The amount of N fixed by berseem clover was estimated as:

$$N_{\text{fixed}} = \text{Total clover } N \times \frac{\%Ndfa}{100}$$

Analysis of variance (procedure ANOVA, SAS Institute, 2004) was performed separately per cut according to the experimental design. All measured variables were assumed to be normally distributed. All variables corresponding to proportions were arcsine transformed before analysis to assure a better fit with the Gaussian law distribution. Treatment means were compared using Tukey's test (P values) at 5% probability level.

## 2.2 EXPERIMENT 2

Where mineral fertilizers can't be used, N availability for plant depends on net N mineralization (Nadelhoffer *et al.*, 1985) and plants compete for N with soil microorganisms, such as soilborne fungi and bacteria. The effect of AM symbiosis on mineralization of organic nitrogen is still unclear. Early studies have shown that AM fungi can improve plant N from organic matter (e.g. Hodge, 2003), but no information is available about its mechanism.

The aim of this experiment was to determine the effects of AM symbiosis on the process of organic N mineralization, on plant N capture and on soil bacterial community. This experiment was funded by the *fondo Ex-60% 2007* "Ruolo della simbiosi micorrizica sull'efficienza di utilizzazione dell'azoto in differenti genotipi di frumento", Università degli Studi di Palermo, Italy; and by the *proyecto AGL2008-00742/AGR*, Ministerio de Ciencia e Innovación (MICINN), Spain.

### 2.2.1 GROWTH CONDITIONS, EXPERIMENTAL DESIGN AND TRATMENT ESTABLISHMENT

The experiment was conducted at Experimental Station of the Zaidín (CSIC) (Granada, Spain) during the winter of 2008 in a conditioned glasshouse, with 25/19 °C day/night temperature, a photoperiod of 16 h. Additional light at a photosynthetic photon flux density of 460  $\mu\text{mol m}^{-2} \text{s}^{-1}$  was provided if necessary.

A complete randomized factorial design replicated 4 times with two factors was adopted. Treatments were (1) AM inoculation: *Glomus mosseae* (T.H. Nicolson and Gerd.) Gerd. and Trappe isolate BEG 12; and not inoculated control; (2) addition of organic matter (OM): soil amended with  $^{15}\text{N}$ -enriched biomass of maize (root or leaves); not amended soil. An identical set of pots with OM treatments and no plant was made in order to evaluate the effects of adding OM on N mineralization processes and bacterial community. For the mycorrhizal treatments, inoculum was obtained from a thoroughly homogenized rhizosphere sample coming from a open-pot culture of *Sorghum bicolor* L. and consisted in soil, spores and mycelia. AM inoculum was added to the appropriate pots at a rate of 1 g inoculum per pot. For the OM treatments:  $^{15}\text{N}$ -enriched organic matter was prepared by a previous maize cultivation on  $^{15}\text{M}$  enriched soil. Maize was harvested at before anthesis and separated in roots, stems and leaves. Fine roots (under ~1 mm diameter) and leaves were used as organic amendant. Roots and leaves were cut in ~1 mm and ~1 mm<sup>2</sup> pieces, respectively. AM colonization of maize root was 17%. N concentrations (in parenthesis, the corresponding  $^{15}\text{N}$  abundance [APC] are given) of maize tissues was 1.90% (4.78% Atom percent PC) and 1.56 (3.94% APC) for leaves and roots, respectively. Organic biomass was oven dried at 80 °C for 1 day before adding to mixture.

Each pot (10 cm diameter, 11 cm height, 72 in total) was filled with 600 g of a quarts sand:soil mixture (2:1). Soil properties were 37% sand, 43% silt, 20% clay; 1.8% organic matter, pH 8.1 (soil:water 1:1); 0.12 mS cm<sup>-1</sup> saturated E.C. (25°C); 1.05‰, 6.2 ppm and 132 ppm of N, P (as P<sub>2</sub>O<sub>5</sub>) and K (as: K<sub>2</sub>O), respectively; 10.06% total Ca; 99



ppm soluble Ca and 16 ppm Mg. Before mixing soil with sand, soil and AM inoculum bacterial microflora were extracted by suspending 500 g soil or 500 g inoculum in 1.5 l distilled water. After shaking and decanting, the suspension was filtered (11  $\mu$ m mesh) in order to discard natural AM fungi. After shaking and decanting, the suspension was filtered (11  $\mu$ m mesh) in order to discard natural AM fungi. Both soil and sand were 2 mm sieved and autoclaved (121°C for 20 min).  $^{15}$ N enriched maize biomass was added at a rate of 4.6 g dry OM per one kg mixture and both OM amended and not amended mixtures were steam sterilized at 95°C for 1 h during three consecutive days in order to completely impair biological (both fungal and bacterial) activity. In order to normalize the differences in the starting microbial community, each pot received 30 ml soil suspension filtrate and 30 ml AM inoculum suspension filtrate before starting the experiment (Koide & Li, 1989; van der Heijden *et al.*, 1998). OM added with AM inoculum was negligible if compared to the quantity added for the OM treatment.

Seeds of wheat (cv. Simeto) were surface sterilized and germinated on wet filter paper in Petri dishes for 3 days. Five seedling were transplanted per each pot and thinned to 3 plants four days after transplanting. During the experiment, each pot received 5 ml modified Hoagland's solution (Hoagland & Arnon, 1950) and 50 ml tap water once per 5 and 3 days, respectively. Modified Hoagland's solution used in the experiment was lacking P and had 10% N strength, only.

One third of the pots (4 pots per each treatments combination) were harvested at 7 weeks after transplanting (WAT), while the others two were harvested at 9 and 13 WAT, respectively. Due to a technical problem of the greenhouse, air temperature reached a maximum daily temperature of 35°C during the last week of the experiment and it resulted in a thermal stress for plants that slightly reduced plant yield. During this week, soil temperature was maintained by giving pot 50 ml water once a day. At each harvest, a sample of soil per pot and total plant biomass were taken. Soil samples were saved at -80°C for further analyses.

### **2.2.2 PLANT ANALYSIS**

Plant biomass was immediately separated into roots, leaves+stems and heads and fresh weights were recorded. Roots were rinsed free of soil, cut into 1-cm fragments and thoroughly mixed. Representative root samples were taken for determination of root AM colonization and fungal alkaline phosphatase (ALP) and succinate dehydrogenase (SDH). Shoots, heads and remaining roots were oven dried at 80 °C for 24 h and dry weight was taken. For measuring AM root colonization, root samples was cleared with 10% (w/v) KOH and stained with 0.05% (v/v) trypan blue using the method described by Phillips & Hayman (1970). For measuring ALP and SDH, root samples were previously cleared (as described by Vierheilig *et al.*, 2005) for 2 h in a solution containing 0.05 M Tris/citric acid (pH 9.2), 0.05% sorbitol, 15 units ml<sup>-1</sup> cellulase and 15 units ml<sup>-1</sup> pectinase (both enzymes were from *Aspergillus niger*). Roots samples were subsequently rinsed in distilled water and placed in the appropriate staining for ALP and SDH as described in Tisserant *et al.* (1993) and Smith & Gianinazzi-Pearson (1990), respectively. Measures of root AM colonization, ALP and SDH fungal activities were made by observing root pieces under the microscope and counting 250-300 total

intersections by the grid intersect method (Giovanetti & Mossee, 1980). Above ground and root plant biomasses were analyzed for total N and  $^{15}\text{N}$  enrichment, using an elemental analyzer – isotope ratio mass spectrometry (EA-IRMS, Carlo Erba NA1500).

### **2.2.3 SOIL ENZYMATIC ACTIVITY, DNA EXTRACTION AND PCR-DGGE ANALYSIS**

Soil enzymatic activities were measured for both wheat-cultivated and uncultivated pots. Four enzymatic activity were measured: dehydrogenase activity (according with García *et al.*, 1997) as an index of microbial activity; casein protease (also referred as ‘casein hydrolizing activity’ or ‘caseinase’, measured according with Ladd & Butler, 1972) as measure of protein hydrolysis to mono- and dipeptides; BAA-protease (according with Nannipieri *et al.*, 1980; and with Tabatabai, 1994) and urease (according with Kandeler & Gerber, 1988) as measure of amino-acids deamination.

The total DNA was extracted from 250 mg of soil samples collected at the last sampling date (13 WAT) and from the bacterial inoculum (4 extractions per each pot) by the bead-beating method, following the manufacturers’ instructions MoBio UltraClean Soil DNA Isolation kit (MoBio Laboratories Inc., Solana Beach, CA, USA) with a few modifications, including the repetition of the second step (Inhibitor Removal Solution) to remove trace concentrations of PCR inhibitors. The DNA samples from each treatments were mixed and checked for concentration and quality using the NanoDrop<sup>®</sup> ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware; USA). PCR was performed with the 16S rRNA universal bacterial primers (TIB<sup>®</sup>MOLBIOL, Berlin, Germany) F341, R907 and GC-F341 (P3) to amplify the V3–V5 hypervariable regions of 16S rRNA genes. Primer P3 contains the same sequence as F341 but with an additional 40-nucleotide GC-rich sequence (GC clamp) at its 5’ end (Yu & Morrison, 2004). The PCR program was initiated by a hot start of 5 min at 94 °C; after 9 min of initial denaturation at 95 °C, a touchdown thermal profile protocol was used, and the annealing temperature was decreased by 1 °C per cycle from 65 °C to 55 °C; then 20 additional cycles at 55 °C were performed. Amplification was carried out with 1 min of denaturation at 94 °C, 1 min of primer annealing, and 1.5 min of primer extension at 72 °C, followed by 10 min of final primer extension. The total reaction mixture of the first PCR consisted of 25 µl with the following ingredients: approx. 1 ng of extracted DNA, 1 µM primer F341, 1 µM primer R907, 0.2mM dNTPs, 5 U Taq polymerase (Bioline GmbH, Germany), 1X PCR buffer (included in the ) and 1.5 mM  $\text{MgCl}_2$ , and sterile Milli-Q water to a final volume of 25 µl. The second amplification was performed by using 1 µl of the products of the first reaction as template. In this amplification, primers P3 and R907 were used under the same conditions as described above. PCR products were analyzed by electrophoresis in 2% agarose gels stained with GelRed<sup>™</sup>.

PCR-DGGE was performed to evaluate the effect of treatments on bacterial community of soil. DGGE analyses were conducted 4 times, using 5, 7, 10 and 15 µl of the latter PCR product, respectively, loaded into a 45–60% urea-formamide–polyacrylamide gradient gel. An INGENYphorU System (Ingeny International BV, The Netherlands) was run at 100 V for 16 h at 59 °C to separate the fragments. Gels were silver stained according to Radojkovica & Kusic (2000) and scanned. Band patterns

from the clearest gel were further analyzed. Band patterns in different DGGE lanes were compared with the UVImap Analysis software (UVItec Limited, Cambridge, UK). The lanes were normalized to contain the same amount of total signal after background subtraction and the gel images were straightened and aligned to give a densitometric curve. Bands were assigned and matched automatically and then checked manually. Band positions were converted to RF values between 0 and 1.

#### 2.2.4 CALCULATIONS AND STATISTICAL ANALYSIS

According to Allen *et al.* (2004), the N Recovery Fraction (%N<sub>REC</sub>; i.e. the N deriving from OM above N applied with OM) of wheat was calculated as follows:

$$N_{REC} = N_t \times \frac{{}^{15}N_a - {}^{15}N_b}{{}^{15}N_c - {}^{15}N_b} \quad ; \quad \% N_{REC} = \frac{N_{REC}}{f} \times 100$$

where  $N_t$  is the total N content (g pot<sup>-1</sup>) in wheat;  ${}^{15}N_a$ ,  ${}^{15}N_b$ , and  ${}^{15}N_c$  are the <sup>15</sup>N isotopic concentration of wheat grown with the organic amendant (either maize leaves or roots), without the organic amendant, and of the organic amendant (either maize leaves or roots), respectively. Finally,  $f$  is the total N of the organic amendant (either maize leaves or roots).

Profile similarity by DGGE lanes was calculated by determining species evenness (E) and abundance (A) as follows:

$$E = n / n_{max} \quad ; \quad A = n / n_{tot}$$

where  $n$  is the number of lines from a given band;  $n_{max}$  is the number of band from the line with most number of bands; and  $n_{tot}$  is the total number of line detected in all DGGE lines (i.e. the sum of all common and uncommon bands).

Nei and Li's similarity coefficients (Nei & Li, 1979) was also calculated for the total number of lane patterns from the DGGE gel and the similarity coefficients calculated were then used to construct a dendrogram using the unweighted pair-group method with arithmetical averages (UPGMA).

DGGE banding data were used to estimate three diversity indices by treating each band as an individual operational taxonomic unit (OTU). For these analyses, each band was presumed to represent the ability of that bacterial species to be amplified (Ibekwe & Grieve, 2004). Each banding pattern was used as a semi-quantitative measure of bacterial diversity (Dilly *et al.*, 2004). The Shannon index of general diversity  $H'$  (Shannon & Weaver, 1963) and the reciprocal Simpson index of dominance  $D_2$  (Simpson, 1949) were calculated from the number of bands present and the relative intensities of the bands in each lane. The similarity coefficients calculated were then used to construct a dendrogram using the unweighted pair-group method with arithmetical averages (UPGMA). Estimates of the number of clusters (soil bacterial communities) were achieved using pseudo  $F$  and  $t^2$  statistics (Milligan & Cooper, 1985). The relevance of the cluster tree was evaluated by computing the cophenetic

correlation coefficient (routine CPH of the NTSYS-pc package). The closer the cophenetic coefficient to 1, the more relevantly the cluster tree reflects the structure of the data. Finally, matrices based on and DGGE banding pattern and enzymatic activities distances were compared calculating the Mantel's test statistic  $Z$  (Mantel, 1967), and significance was determined using 1000 permutations (routine MXCOMP of the NTSYS-pc package; Rohlf, 1998).

Moreover, Shannon index of equitability  $E_H$  was calculated by dividing  $H'$  by  $\ln S$ . The intensity of the bands was reflected as peak heights in the densitometric curve. The Shannon  $H'$  and Simpson  $D_2$  indexes were calculated from the following equations:

$$H' = -\sum (P_i * \log P_i); \quad D_2 = 1 / \sum P_i^2$$

where  $P_i = n_i/N$ ;  $n_i$  is the height of peak and  $N$  is the sum of all peak heights in the curve.

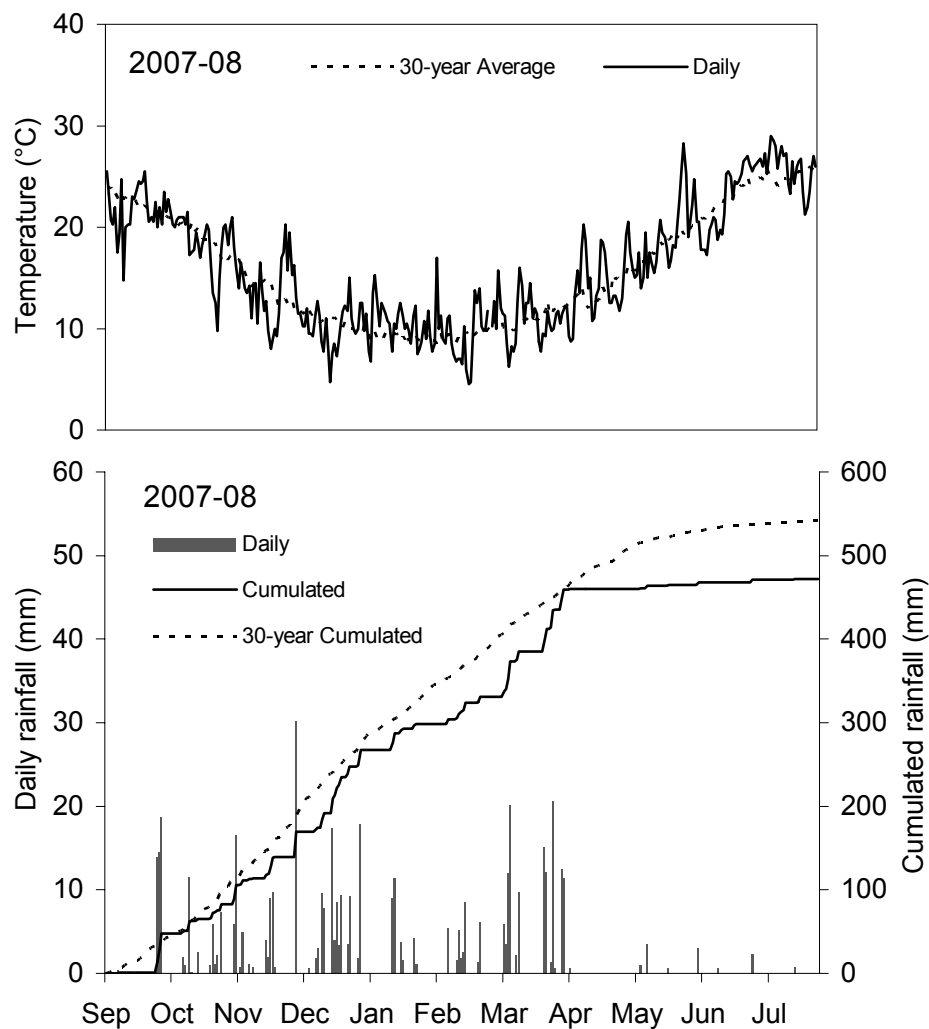
DGGE bands can be considered as the ability of that bacterial taxonomic unit to be amplified (Ibekwe & Grieve, 2004) and Dilly *et al.* (2004) proposed DGGE banding pattern as a semi-quantitative measure of bacterial diversity. To test if the similarities observed within and between samples were greater or less than those expected by chance, band matching data were stored as a binary matrix and analyzed using Raup and Crick's probability-based index of similarity  $S_{RC}$  (Raup & Crick, 1979). The  $S_{RC}$  is the probability that the randomized similarity would be greater than or equal to the observed similarity, and  $S_{RC}$  values above 0.95 or below 0.05 signify similarities and differences, respectively, which are not random assortments of the same species (bands or OTUs) (Rowan *et al.*, 2003).  $S_{RC}$  was calculated using the PAST (Palaeontological statistics, version 1.97) program (Hammer *et al.*, 2001).

Data on plant production, quality, root AM infection and activity and on soil enzymatic activities were subjected to analysis of variance (ANOVA) according to the experimental design. Data of enzymatic activity from unplanted and wheat-cultivated pots analyzed separately. All measured variables were assumed to be normally distributed. All variables corresponding to proportions were arcsine transformed before analysis to assure a better fit with the Gaussian law distribution. Treatment means were compared using Tukey's test (P values) at 5% probability level.

### 3 RESULTS AND DISCUSSION

#### 3.1 EXPERIMENT 1

Total rainfall during the growing season was 13% lower than the long term average (551 mm). During spring, rainfalls were markedly lower (–56%) than normal and it caused drought stressing conditions for the crop. Average, minimum and maximum temperatures were similar to long-term averages (Fig. 3-1).



*Fig. 3-1 – Daily average air temperature and daily and cumulated rainfall during the 2007–2008 growing season at the study area in Pietranera field station (37°33'N – 13°30'E, 170 m a.s.l.), Sicily, Italy.*

### **3.1.1 BIOMASS PRODUCTION AND ROOT COLONIZATION BY AM FUNGI AT THE FIRST AND SECOND CUT**

At 55 DAS, mycorrhizal infection in +AM treatments was 32.4% and 23.6% for berseem and ryegrass, respectively. The fungicides applied negatively affected the root infection by AM fungi. Root AM colonization in –AM treatments was 7.0% in berseem and 1.7% in ryegrass. These results accord with reports from pot and field studies by several authors (Dodd & Jeffries, 1989; Sukarno *et al.*, 1993; Udaiyan *et al.*, 1995).

At 76 DAS (first cut), root dry matter (DM) yield, aboveground biomass (both removed and residual), and respective Leaf Area Indices (LAIs) of berseem were significantly higher in the +AM than the –AM treatment, whereas, at 116 DAS (second cut) no significant effect of mycorrhization treatment were observed (Tab. 3-1).

Given C limitation induced by the detopping of berseem, the advantages of the AM symbiosis were probably neutralized by the C cost of the AM fungi. Koide & Elliot (1989) and Kiers & Denison (2008) found that plants should suppress symbiotic association if the benefits are smaller than the C costs. On the other side, Goss & de Varennes (2002) and Ganry *et al.* (1985) argued that AM symbiosis was important in nutrient uptake in the early phase of the crop cycle, when roots are not well developed and still have a low nutrient absorbing ability. This could explain why the effects of AM treatments were significant only at the first cut. In contrast with results from the present study, Barea *et al.* (1987) found no effect of inoculation of AM fungi on *Hedysarum coronarium* plants at the first harvest, but in the subsequent regrowths AM inoculation significantly enhanced dry matter yield comparing with not inoculated plants. AM infection didn't affect ryegrass production and plant traits both at the first and second cut. Kaschuk *et al.* (2010) reported that yield response of legumes to AM infection in field varies with the plant species and range from yield depression to yield improvement.

Tab. 3-1 – Berseem above ground (removed and residual) and root biomass and ryegrass above ground removed biomass, LAIs and % leaves in mycorrhizal inoculated (+Myc) mycorrhizal depressed (-Myc) crops at 76 (first cut) and 116 (second cut) DAC. Values are means for 8 replicates. Significance of treatments is given: ns, non significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

		First cut			Second cut		
		- Myc	+ Myc		- Myc	+ Myc	
Berseem ( <i>Trifolium alexandrinum</i> )							
Removed above ground biomass	g DM m <sup>-2</sup>	113	136	**	393	410	ns
LAI		2.51	2.87	*	4.62	4.97	ns
% leaves		74.7	68.2	**	40.6	41.5	ns
Residual above ground biomass	g DM m <sup>-2</sup>	46	51	*	155	158	ns
LAI		0.44	0.51	*	1.31	1.33	ns
% leaves		32.2	32.8	ns	28.6	28.5	ns
Root biomass	g DM m <sup>-2</sup>	35	41	*	36	36	ns
Ryegrass ( <i>Lolium multiflorum</i> )							
Removed above ground biomass	g DM m <sup>-2</sup>	45	45	ns	383	391	ns
LAI		1.0	1.0	ns	4.53	4.49	ns
% leaves		100.0	100.0	ns	51.0	46.8	ns

### 3.1.2 EFFECTS OF AM INOCULATION ON BERSEEM PRODUCTION AND SYMBIOTIC N<sub>2</sub> FIXATION UNDER DIFFERENT WATER REGIMES

After the second cut, the effects of AM symbiosis was studied on berseem clover grown in the field under both drought and well-watered conditions as described in the section 2.1.2.

As expected, soil moisture contents, measured at 7, 14, 21, and 28 days after the second cut (layers 0-20 cm and 20-40 cm) were always significantly higher in the well-watered than the drought-stressed conditions (Fig. 3-2). Soil moisture contents weren't affected by AM treatment and interaction between water regime and AM treatment has never been found.

At 14 DAC, AM infection was on average 8.8% and 37.7% in the –AM and +AM treatments, respectively, and no effect of the soil moisture regime treatment was observed. At 28 DAC, root AM colonization was very low in the –AM treatments irrespective of soil moisture regime (8.7% on average). In +AM treatments, AM infection was significantly higher in the drought-stressed than well waters treatments (66.0% and 52.4%, respectively). Such result is consistent with findings from pot studies (e.g. Azcón *et al.*, 1988, for alfalfa), while other authors (Meddich *et al.*, 2000) have observed a higher AM infection in well-watered than drought-stressed berseem. However, in the latter experiment, drought was more severe than in our research. I

hypothesize that the effects of drought vary with its intensity: in moderate stress conditions, the plant favours AM infection to take advantage of the symbiosis, while when drought is severe, probably the plant lacks of photosynthates to feed AM fungi and so AM infection is reduced.

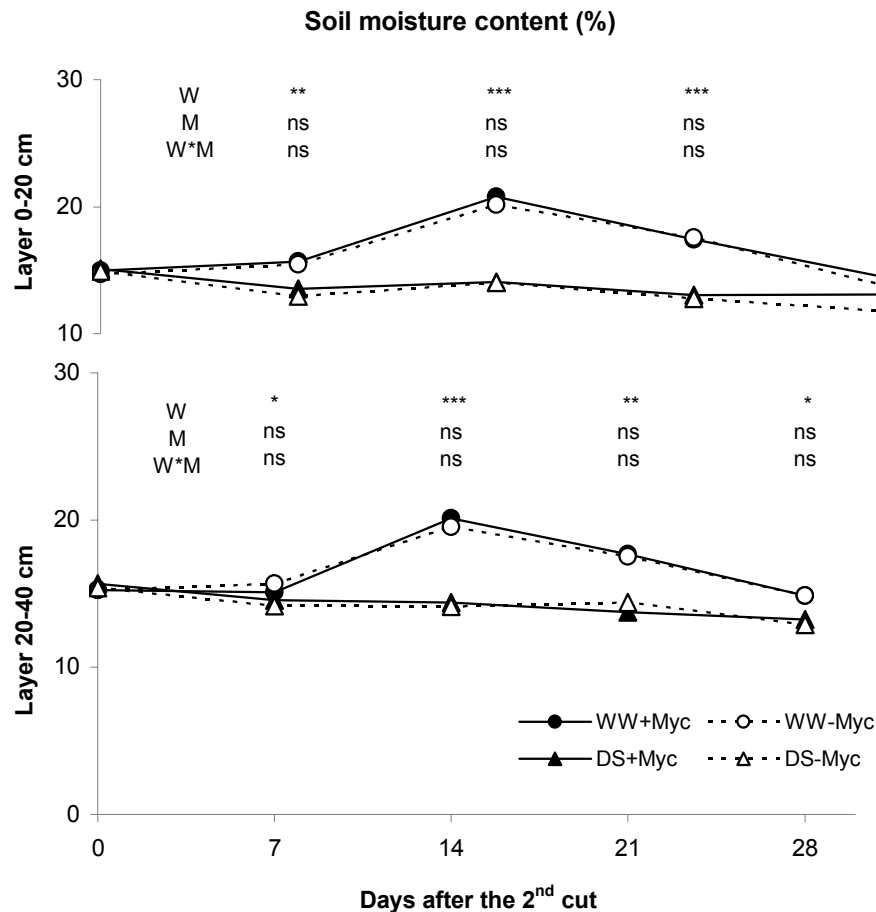


Fig. 3-2 – Effects of treatments on soil moisture content (0 to 20 cm and 20 to 40 cm depth) during regrowth after the second cut. Values are means for 4 replicates. WW, DS for well-watered and drought-stressed plots, respectively; +Myc, and –Myc for mycorrhizal inoculated mycorrhizal depressed plots, respectively. Significance of treatments per each cut is given (W, soil moisture treatment; M, crop mycorrhization treatment, W\*M, interaction): ns, non significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .



The effects of treatments on berseem above ground (AG) biomass during regrowth after the second cut is shown in Fig. 3-3. Since from 7 DAC, berseem biomass was significantly higher under well-watered than drought-stressed conditions. The differences determined by water regime markedly increased at 14 DAC and remained unvaried at 21 and 28 DAC.

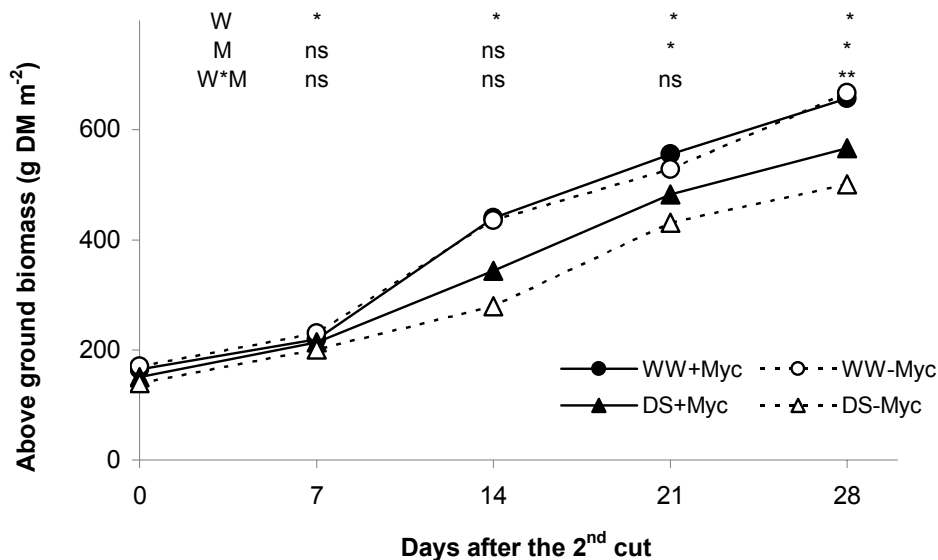


Fig. 3-3 – Effects of mycorrhization and soil moisture regime on berseem above ground biomass during regrowth after the second cut. Values are means for 4 replicates. WW, DS for well-watered and drought-stressed crops, respectively; +Myc, and –Myc for mycorrhizal inoculated mycorrhizal depressed crops, respectively. Significance of treatments per each cut is given (W, soil moisture treatment; M, crop mycorrhization treatment, W\*M, interaction): ns, non significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

AM symbiosis didn't affect AG biomass at 7 and 14 days after the second cut (DAC) both under well-watered and drought-stressed conditions. It is probable that the benefits of AM symbiosis during the first regrowth phase were impaired due to a competition for carbon between AM fungi and shoots. Indeed, during this phase, legume regrowth ability is highly dependent on the plant's capacity to mobilize C and N reserves stored in roots and stubble (Ta *et al.*, 1990; Ourry *et al.*, 1994; Volenec *et al.*, 1996; Avice *et al.*, 1997) and on residual leaf area (Meuriot *et al.*, 2004) to rebuild its photosynthetic apparatus and the high sink strength of the AG parts of the plant could have limited the resources available for the AM fungi, and it didn't produce any growth benefit. This hypothesis is confirmed by the fact that at 21 and 28 DAC, when plants have already rebuilt its photosynthetic apparatus to satisfy both shoots and AM fungi C demands, a higher AG biomass was observed in +AM than –AM treatment.

The advantages of the AM symbiosis were higher in drought-stressed than well-watered conditions, particularly at 28 DAC. This result is consistent with findings of

several pot studies (Azcón *et al.*, 1988; Ruiz-Lozano *et al.*, 1995; Marulanda *et al.*, 2003; Porcel & Ruiz-Lozano, 2004). Several authors reported that the AM contribution to plant drought tolerance are related to drought avoidance mechanisms such as hyphal water uptake (Ruiz-Lozano *et al.*, 1995) and enhanced plant osmotic adjustment (Goicoechea *et al.*, 1998; Kubikova *et al.*, 2001) that favour plant water uptake from soil. Some authors (Ruiz-Lozano *et al.*, 1996; Ruiz-Lozano *et al.*, 2001b) suggested that the advantages of the AM symbiosis in drought stressing conditions are related to a protection effect of AM fungi against oxidative damages generated by drought (Bartels, 2001). However, other authors (Al-Karaki *et al.*, 2004) observed a beneficial effect of an increased AM infection to yield and quality of wheat grown in field both in well-watered and drought-stressed conditions.

Both treatments applied (water regime and mycorrhization) didn't significantly affect berseem root biomass in all sampling dates (Fig. 3-4). Other researches (Hawkins & George, 1999; Li *et al.*, 2006) have found no differences in root dry weight due to the presence of the AM symbiosis. The hypogeic:epigeic biomass ratio was higher in plants grown in drought than well-watered conditions, and it wasn't affected by the AM treatment (Fig. 3-5).

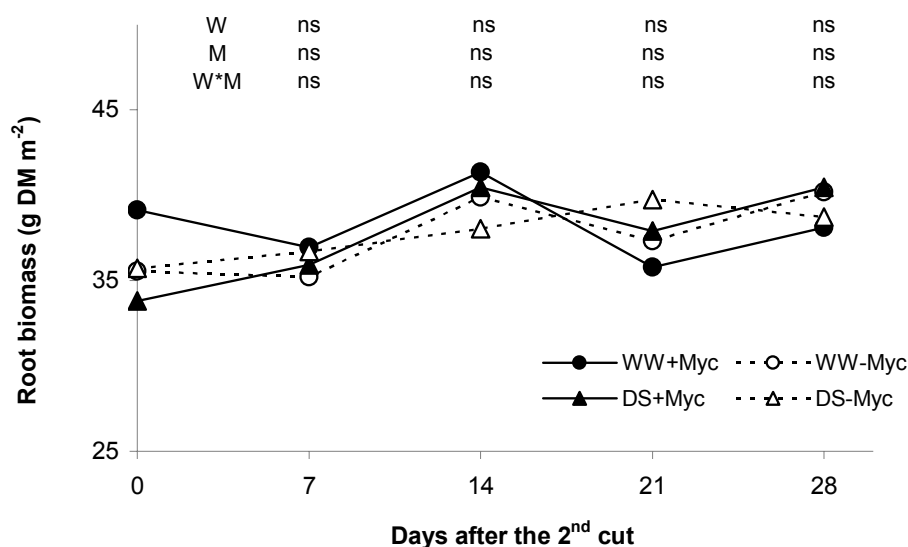


Fig. 3-4 – Effects of mycorrhization and soil moisture regime on berseem root during regrowth after the second cut. Values are means for 4 replicates. WW, DS for well-watered and drought-stressed crops, respectively; +Myc, and –Myc for mycorrhizal inoculated mycorrhizal depressed crops, respectively. Significance of treatments per each cut is given (W, soil moisture treatment; M, crop mycorrhization treatment, W\*M, interaction): ns, non significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$

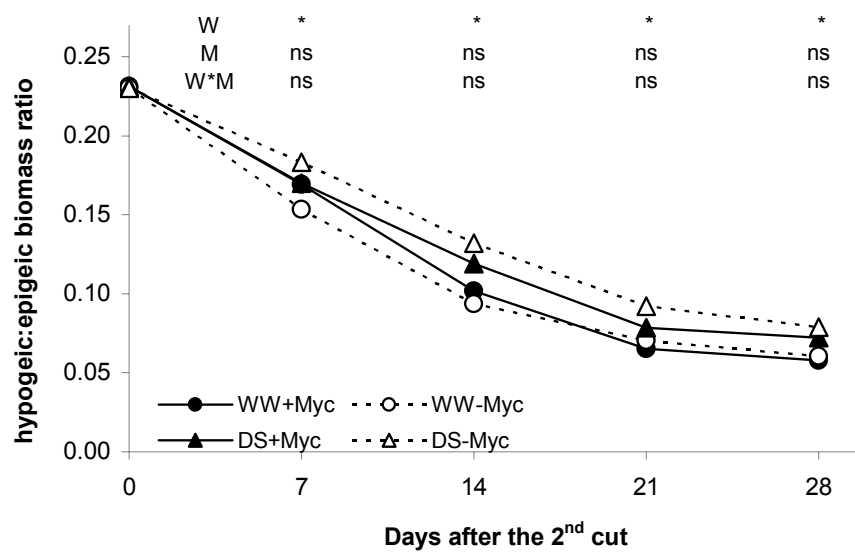


Fig. 3-5 – Effects of mycorrhization and soil moisture regime on hypogeic:epigeic biomass ratio of berseem during regrowth after the second cut. Values are means for 4 replicates. WW, DS for well-watered and drought-stressed crops, respectively; +Myc, and –Myc for mycorrhizal inoculated mycorrhizal depressed crops, respectively. Significance of treatments per each cut is given (W, soil moisture treatment; M, crop mycorrhization treatment, W\*M, interaction): ns, non significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

A higher leaves, but lower stems and heads percentages were observed in biomass of berseem grown under well-watered than drought-stressed conditions (Fig. 3-6, Fig. 3-7, Fig. 3-8 for leaves, stems and heads, respectively). The differences in stems and heads percentages due to the soil moisture content increased during time. Crop mycorrhization never affected the proportion of the botanical fractions on the epigeic biomass of berseem.

Leaf area index was significantly higher in the well-watered than drought-stressed crop (Fig. 3-9), the highest LAI values were observed at 21 days after the second cut (5.42 and 2.88, respectively). For this trait, no significant effects of crop micorrhization were observed, as well.

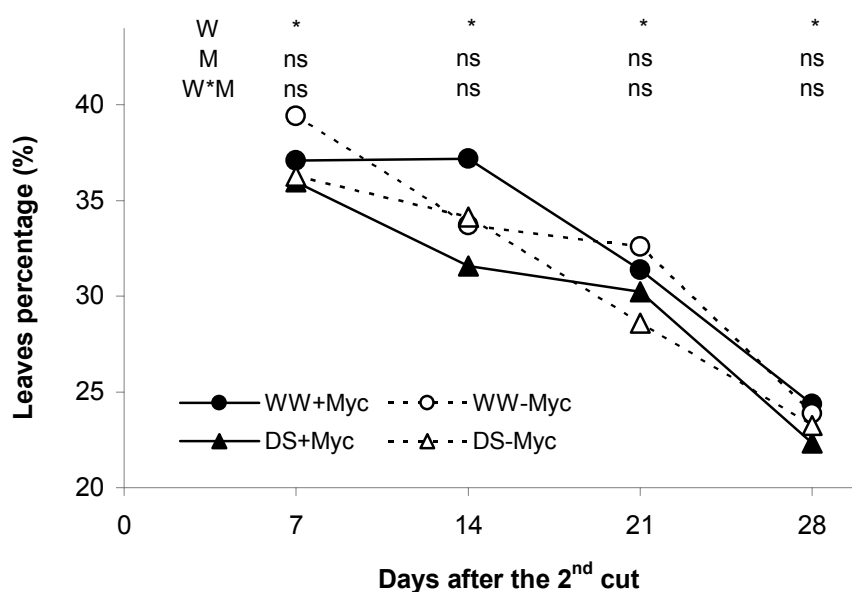


Fig. 3-6 – Effects of mycorrhization and soil moisture regime on leaves percentage of berseem during regrowth after the second cut. Values are means for 4 replicates. See Fig. 3-5 for symbols and abbreviations.

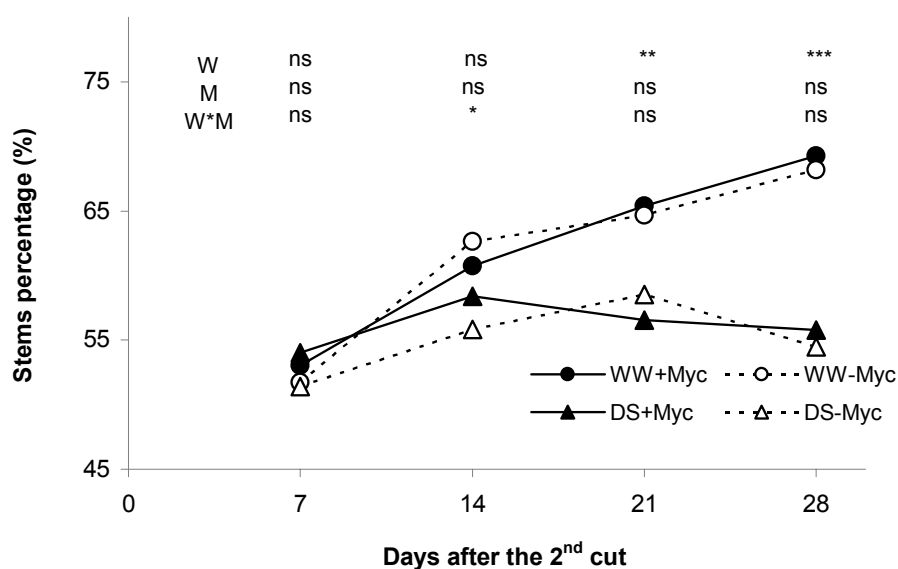


Fig. 3-7 – Effects of mycorrhization and soil moisture regime on leaves percentage of berseem during regrowth after the second cut. Values are means for 4 replicates. See Fig. 3-5 for symbols and abbreviations.

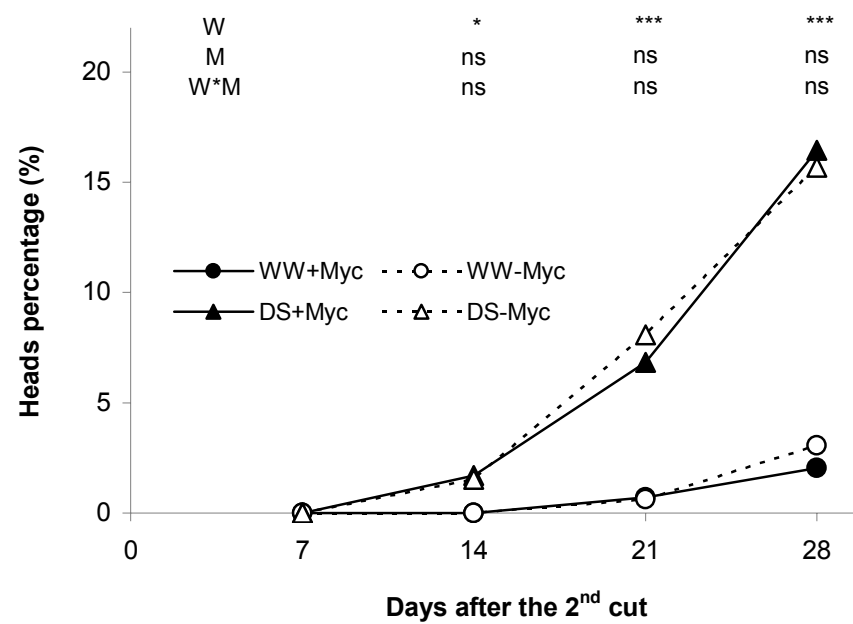


Fig. 3-8 – Effects of mycorrhization and soil moisture regime on heads percentage of berseem during regrowth after the second cut. Values are means for 4 replicates. See Fig. 3-5 for symbols and abbreviations.

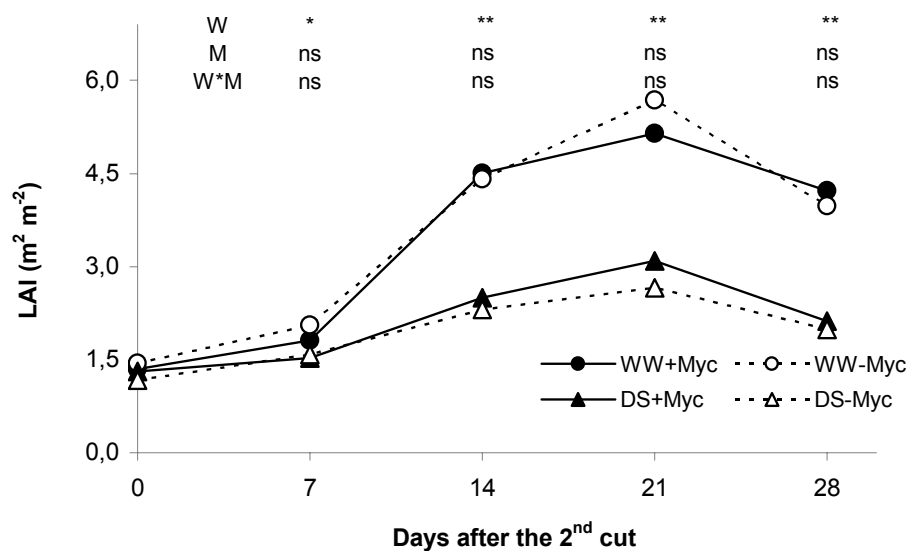


Fig. 3-9 – Effects of mycorrhization and soil moisture regime on LAI of berseem during regrowth after the second cut. Values are means for 4 replicates. See Fig. 3-5 for symbols and abbreviations.

Tab. 3-2 – N concentration, N uptake and Ndfa of berseem at 14 and 28 days after the second cut in crops grown under well-watered and drought-stressed (rainfed) conditions. +Myc, and –Myc for mycorrhizal inoculated mycorrhizal depressed crops, respectively. Each value is a mean for 4 replicates. Significance of treatments is given: ns, non significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

			Drought-stressed		Well-watered		W	M	W*M
			- Myc	+ Myc	- Myc	+ Myc			
Measures at 14 days after the 2 <sup>nd</sup> cut									
N concentration									
above ground N	%		3.1	3.1	3.1	3.0	ns	ns	ns
root N	%		1.8	1.8	1.9	1.9	ns	ns	ns
N uptake									
above ground N	g N m <sup>-2</sup>		9.2	10.6	13.1	13.1	*	ns	ns
root N	g N m <sup>-2</sup>		0.6	0.7	0.8	0.8	**	ns	ns
Ndfa									
percentage			49.9	49.9	60.7	56.4	*	ns	ns
amount	g N m <sup>-2</sup>		4.4	5.7	7.8	7.3	**	ns	ns
Measures at 28 days after the 2 <sup>nd</sup> cut									
N concentration									
above ground N	%		2.5	2.4	2.5	2.5	ns	ns	ns
root N	%		1.8	1.8	1.8	1.8	ns	ns	ns
N uptake									
above ground N	g N m <sup>-2</sup>		12.3	13.7	16.9	16.2	*	ns	*
root N	g N m <sup>-2</sup>		0.7	0.7	0.7	0.7	ns	ns	ns
Ndfa									
percentage			40.4	52.9	50.3	52.7	*	ns	*
amount	g N m <sup>-2</sup>		5.2	7.6	8.9	8.9	*	*	*

N concentration in AG and root biomass of berseem and total N uptake didn't vary with crop mycorrhization both at 14 and 28 DAC, except at 28 DAC where AM symbiosis enhanced total N uptake in the drought-stressed crop, only (Tab. 3-2). Such results are consistent with findings by other authors (Hawkins & George, 1999; Aliasgharзад *et al.*, 2006). On the contrary, Azcón & Al-Atrash (1997) showed that AM symbiosis increased N concentration and N uptake in *M. sativa* of almost two- and threefold, respectively, comparing to the non-mycorrhizal control. Soil moisture regimes didn't affect N concentration, but, given its effect on DM yield, it markedly influenced total N uptake at both 14 and 28 DAC.

Ndfa (as both as percentage of total N uptake and the amount of N fixed) was significantly higher in well-watered than in drought-stressed conditions at both 14 and 28 DAC. Several researches showed that symbiotic N<sub>2</sub> fixation of legumes is highly sensitive to soil water deficiency (as reviewed by Zahran, 1999). This occurs because nodule activity is more sensitive to drought than shoot and root metabolism (Albrecht *et al.*, 1994). Results from the present research seem to confirm this assumption; in fact the reduction of symbiotic N<sub>2</sub> fixation caused by drought was higher than DM yield.

At 14 DAC, no effect of crop mycorrhization was observed on N<sub>2</sub> fixation, but at 28 DAC, a higher Ndfa was observed for +AM than –AM in the drought-stressed (rainfed) conditions, only. Many authors stated that N<sub>2</sub> fixation can be improved by AM symbiosis, resulting in a higher amount of N fixed and in a higher proportional dependence of the legume on atmospheric N<sub>2</sub> (Schoeneberger *et al.*, 1989; Olesniewicz & Thomas, 1999; Goss & de Varennes, 2002; Antunes *et al.*, 2006b) and in many cases such results were attributed to the benefits of AM fungi on improved P acquisition (Barea & Azcón, 1983).

However very few data exist on the interactive effects of AM symbiosis and environmental factors on the performance of the tripartite symbiosis and particularly on the amount of symbiotic N<sub>2</sub> fixation. Porcel *et al.* (2003) found that N<sub>2</sub>ase activity increased more in AM than non-AM soybeans in a controlled environment and that this increase was more evident under drought-stressed conditions. Ruiz-Lozano *et al.* (2001a) found that AM symbiosis was able to reduce nodule senescence under drought stress conditions and attributed this effect to a protection against oxidative damages which have detrimental effects on N<sub>2</sub>ase activity.

### **3.1.3 BIOMASS PRODUCTION, QUALITY AND ROOT AM INFECTION OF RYEGRASS UNDER DIFFERENT SOIL MOISTURE REGIMES**

Data about biomass production, quality and root AM infection of annual ryegrass at 14 and 28 DAC are given in Tab. 3-3.

Fungicide applied strongly depressed AM infection in both sampling moments. Root AM colonization of +AM treatments was significantly lower under well-watered than drought-stressed condition at both 14 and 28 DAC as already observed in berseem. Similar results were found by Ruiz-Lozano *et al.* (1995) for lettuce infected by *G. mosseae* and by Manske *et al.* (1995) in wheat. However, other authors found depressive (Al-Karaki *et al.*, 2004) or null effects (Ruiz-Lozano *et al.*, 1996) of drought stress on root AM colonization in non leguminous crops. As for berseem, here I hypothesize that the effects of drought vary with its intensity. In addition, it seems that the effect of water regime on root AM colonization of non-leguminous species seems to vary with the AM fungus strain: for example, Ruiz-Lozano *et al.* (1995) found a higher AM colonization in drought-stressed than well-watered lettuce inoculated by *G. mosseae*, but not with other AM fungi. In the present research, it is probable that plant stimulated AM infection in a higher extent under drought-stressed than well-watered conditions in order to overcome the stressing effect of water deficiency. However, plant growth was surely impaired by the lack of N fertilization irrespective of the water regime and mycorrhization treatments. This is clearly visible considering that plant N concentration at both 14 and 28 DAC was almost a half if comparing with N fertilized ryegrass grown in similar conditions (Giambalvo *et al.*, 2005).

A higher water availability resulted in both higher AG biomass and N uptake at 14 and 28 DAC and it surely depended by the higher transpiration rates under well-watered than drought-stressed conditions, as also shown by Yin *et al.* (2009). No differences on root biomass have been found under the different water regimes.

Tab. 3-3 – Root AM colonization, above ground and root biomass, LAIs, % of leaves, stems and spikes, hypogeic:epigeic biomass ratio and N concentration and uptake of ryegrass at 14 and 28 days after the second cut in crops grown under well-watered and drought-stressed (rainfed) conditions. +Myc, and –Myc for mycorrhizal inoculated mycorrhizal depressed crops, respectively. Each value is a mean for 4 replicates. Significance of treatments is given: ns, non significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

		Drought-stressed		Well-watered		W	M	W*M
		- Myc	+ Myc	- Myc	+ Myc			
<b>Measures at 14 days after the 2<sup>nd</sup> cut</b>								
Root AM colonization	%	7.6	35.5	3.0	30.9	*	*	ns
Above ground biomass	g DM m <sup>-2</sup>	229	230	379	406	***	ns	ns
% leaves	%	15.9	18.8	14.8	19.0	ns	*	ns
% stems	%	17.1	19.0	16.4	17.8	ns	ns	ns
% spikes	%	0.8	0.5	0.3	0.0	ns	ns	ns
LAI		1.30	1.26	2.57	2.80	*	ns	ns
Root biomass	g DM m <sup>-2</sup>	29	36	33	45	ns	***	ns
Hypogeic:epigeic biomass ratio		0.13	0.16	0.09	0.11	*	*	ns
N concentration								
above ground N	%	1.86	1.85	1.88	1.59	ns	ns	ns
root N	%	0.72	0.76	0.65	0.62	*	ns	ns
N uptake								
above ground N	g N m <sup>-2</sup>	4.26	4.26	7.13	6.46	**	ns	ns
root N	g N m <sup>-2</sup>	0.22	0.28	0.21	0.28	ns	**	ns
<b>Measures at 28 days after the 2<sup>nd</sup> cut</b>								
Root AM colonization	%	11.5	49.2	11.2	37.0	*	*	*
Above ground biomass	g DM m <sup>-2</sup>	376	348	532	526	*	ns	ns
% leaves	%	20.1	20.0	19.0	18.2	ns	ns	ns
% stems	%	18.7	18.5	17.4	20.6	ns	ns	ns
% spikes	%	14.5	15.6	14.9	15.7	ns	ns	ns
LAI		1.60	1.43	3.15	3.36	*	ns	ns
Root biomass	g DM m <sup>-2</sup>	39	44	40	45	ns	*	ns
Hypogeic:epigeic biomass ratio		0.11	0.13	0.08	0.09	*	ns	ns
N concentration								
above ground N	%	1.73	1.40	1.66	1.30	ns	*	ns
root N	%	0.83	0.76	0.76	0.61	ns	ns	ns
N uptake								
above ground N	g N m <sup>-2</sup>	6.50	4.87	8.83	6.84	*	*	ns
root N	g N m <sup>-2</sup>	0.32	0.33	0.31	0.28	ns	ns	ns



AM symbiosis significantly enhanced root biomass irrespective of the soil moisture regime in both samplings. It's usually believed that AM fungi impair root development by competing for C. However, other authors showed that AM can improve root growth, especially in nutrient limited environments (Behl *et al.*, 2003; Schroeder & Janos, 2005; Li *et al.*, 2006). In the present experiment, the lack of N or P fertilizers limited plant growth. In such conditions, AM fungi could have promoted root branching so resulting in a higher root biomass (Atkinson *et al.*, 1994). The presence of the AM symbiosis had no effects on the above ground biomass at both 14 and 28 DAC. In agreement with the present results, several authors found that AM symbiosis can result in no positive AG growth responses at vegetative stages (Simpson & Daft, 1990; Baon *et al.*, 1992; Graham & Abbott, 2000; Zhu *et al.*, 2001; Ryan & Graham, 2002; Ryan & Angus, 2003; Li *et al.*, 2005 and 2006).

N concentration and uptake at 28 DAC was significantly lower in AM than non AM plants. Also Wallace *et al.* (1982) found a lower N uptake in plants subjected to clipping and attributed this effect to the competition for N and C between roots and AM fungi, especially when plants were grown under limiting N availability. Ganry (1982) found that AM symbiosis reduced the uptake of both fertilizer and soil N in a field experiment, while Hodge (2003) showed that AM fungi lowered N capture by *Plantago lanceolata* and *L. perenne* in monocultures and attributed this effects to the interplant competition for N. However, some reports showed that AM symbiosis promote N uptake by plants grown in N limiting (Azcón *et al.*, 2008) or drought-stressed conditions (Goicoechea *et al.*, 1997), while other authors found no positive effects of AM symbiosis on N uptake of plants grown under low N availability (Hawkins & George, 1999) or after defoliation (Hokka *et al.*, 2004). It seems that the contribution of AM fungi to N uptake becomes negligible when roots and hyphae exploit a common space (Hodge *et al.*, 2000b; Mäder *et al.*, 2000).

## 3.2 EXPERIMENT 2

### 3.2.1 RESULTS

Significances of data of wheat production and quality are shown in Tab. 3-4. OM addition significantly affected root AM infection (Fig. 3-10), alkaline phosphatases (ALP, Fig. 3-19) and succinate dehydrogenase (SDH, Fig. 3-12) activities in all samplings. Roots AM colonization was on average 23,2%, 34,9% and 33,3% at 7, 9 and 13 weeks after transplanting (WAT), respectively. ALP activity at the first (7 WAT) and second (9 WAT) sampling date was higher in plants grown in amended soils. At the third sampling (13 WAT), ALP activity of plants grown in root amended soil was lower than plants grown in leaves amended or not amended soils. SDH activity in mycorrhizal roots was enhanced at 7, but depressed 9 and 13 WAT, by the addition of organic matter to soil.

Fig. 3-13 and Fig. 3-14 show wheat epigeic and root biomass, respectively. At the 3<sup>rd</sup> sampling, root biomass decreased of almost 50% irrespective of treatments due to a thermal stress during the last week of growth, but the effect of AM symbiosis and OM addition to plant growth and N uptake were similar to those observed at the 1<sup>st</sup> and 2<sup>nd</sup> cut. AM symbiosis improved above ground plant growth in all sampling, except in wheat grown in root amended pot at the 7 and 9 WAT. Addition of organic matter lowered above ground and root biomass both in AM and non AM treatments. In particular, addition of maize roots to soil consisted in higher depressive effects than addition of maize leaves to soil, especially at the second (7 WAT) and third (9 WAT) samplings. AM symbiosis didn't affect root:epigeic biomass ratio (R/S, Fig. 3-15) On average, R/S (Fig. 3-15) was higher plants grown in not amended that amended soil. An interaction between plant inoculation and OM addition was found. At the first and second sampling dates, R/S of plants grown in root amended and not amended soil was higher in non AM than AM plants, but didn't affect R/S of plants in soil amended with maize leaves. At the third sampling date, AM symbiosis affected R/S in leaves amended soil, only.

No differences in root and shoot N concentration were found among treatments applied (Tab. 3-4). On average, shoot N concentration was 1.36%, 1.13% and 1.23% at the first, second and third sampling date, respectively, while root N concentration was 0.59%, 0.53% and 0.71%, respectively. Differences observed in total N uptake among treatments were similar to those observed in plant growth (data not shown). AM symbiosis in wheat significantly lowered both N recovery fraction (%N<sub>REC</sub>) (Fig. 3-16) and the percentage of plant N deriving from organic matter in comparison with non mycorrhizal treatments (Fig. 3-17).

Tab. 3-4 – Significances of treatments (OM, Myc, OM\*Myc for organic matter addition, AM inoculation treatments and interaction, respectively) at 7, 9 and 13 weeks after transplanting on root AM colonization and activity, wheat epigeic and root biomass production, N concentration and uptake, N recovery fraction ( $N_{REC}$ ) and percentage of plant N deriving from OM. ns, non significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

	df	7 weeks after				9 weeks after				13 weeks after			
		Myc	OM	Myc * OM	OM	Myc	OM	Myc * OM	OM	Myc	OM	Myc * OM	OM
AM infection		-	***	-	-	-	***	-	-	-	ns	-	-
ALP activity		-	***	-	-	-	***	-	-	-	***	-	-
SDH activity		-	***	-	-	-	***	-	-	-	**	-	-
Total biomass	g pot <sup>-1</sup>	**	***	**	***	ns	***	***	***	*	***	ns	ns
Epigeic biomass	g pot <sup>-1</sup>	**	***	*	***	ns	***	***	***	*	**	ns	ns
Root biomass	g pot <sup>-1</sup>	**	***	***	***	ns	***	***	***	*	***	***	***
Root/Epigeic biomass ratio	g g <sup>-1</sup>	ns	***	**	***	ns	***	***	***	ns	**	**	**
Epigeic N concentration	%	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Root N concentration	%	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Total N uptake	mg pot <sup>-1</sup>	*	***	ns	***	ns	***	*	***	**	*	ns	ns
N uptake in epigeic biomass	mg pot <sup>-1</sup>	**	**	ns	ns	ns	**	*	***	**	*	ns	ns
N uptake in root biomass	mg pot <sup>-1</sup>	ns	***	**	***	*	***	*	***	***	***	***	***
N recovery fraction	%	***	***	***	***	***	ns	*	***	***	*	ns	ns
% of plant N deriving from OM	%	***	***	***	***	***	ns	ns	***	***	***	*	*

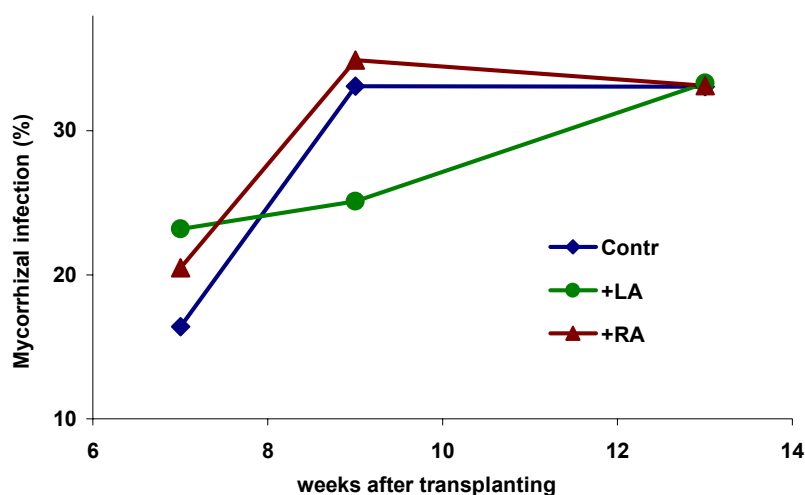


Fig. 3-10 – Percentage of root AM colonization of wheat grown in soil amended with maize leaves (+LA, green lines) or maize roots (+RA, red lines) or non amended soil (Contr, blue lines) at 7, 9 and 13 weeks after transplanting.

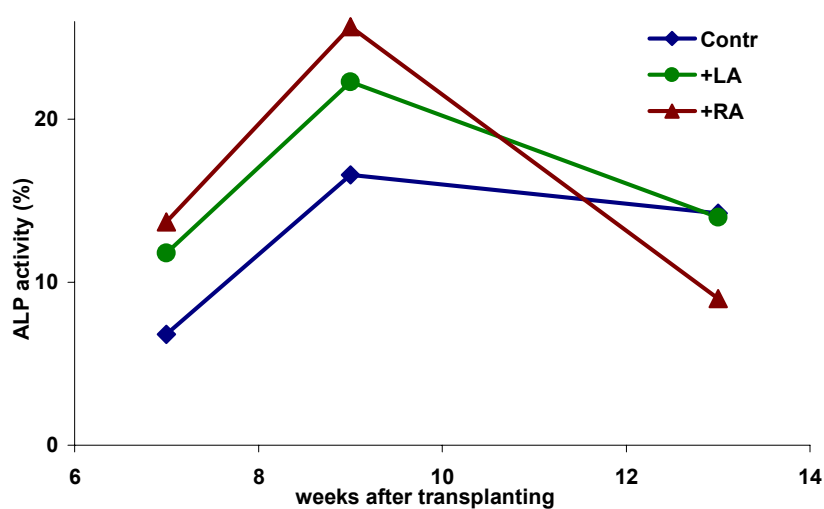


Fig. 3-11 – Root AM colonization percentage after alkaline phosphatase (ALP) staining of wheat grown in soil amended with maize leaves (+LA, green lines) or maize roots (+RA, red lines) or non amended soil (Contr, blue lines) at 7, 9 and 13 weeks after transplanting.

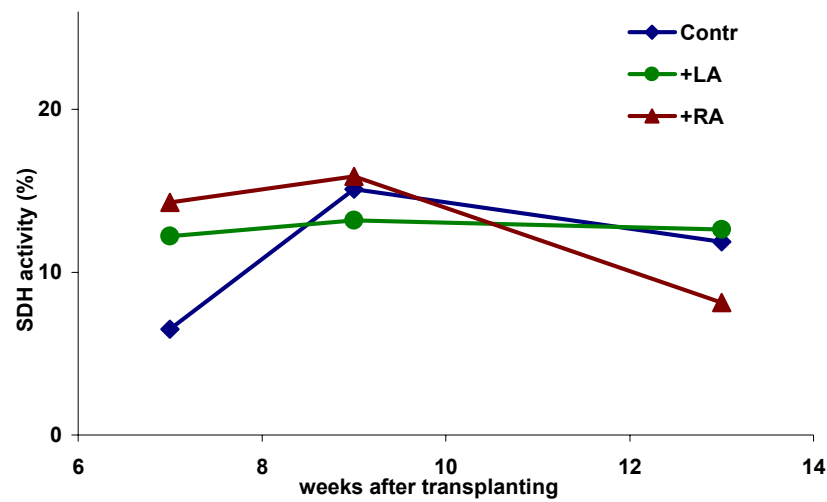


Fig. 3-12 – Root AM colonization percentage after succinate dehydrogenase (SDH) staining of wheat grown in soil amended with maize leaves (+LA, green lines) or maize roots (+RA, red lines) or non amended soil (Contr, blue lines) at 7, 9 and 13 weeks after transplanting.

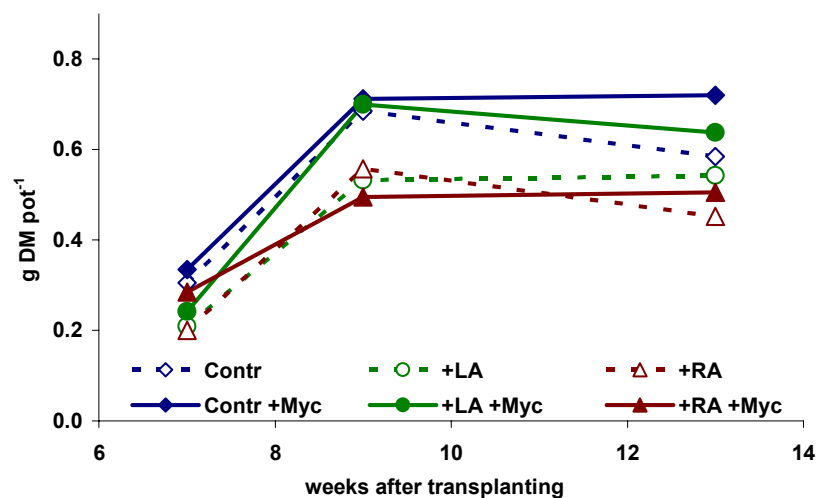


Fig. 3-13 – Epigeic biomass of wheat grown in soil amended with maize leaves (+LA, green lines) or maize roots (+RA, red lines) or non amended soil (Contr, blue lines) and infected with *G. mosseae* (+Myc, continuous lines with closed symbols) or not (dashed lines with open symbols) at 7, 9 and 13 weeks after transplanting.

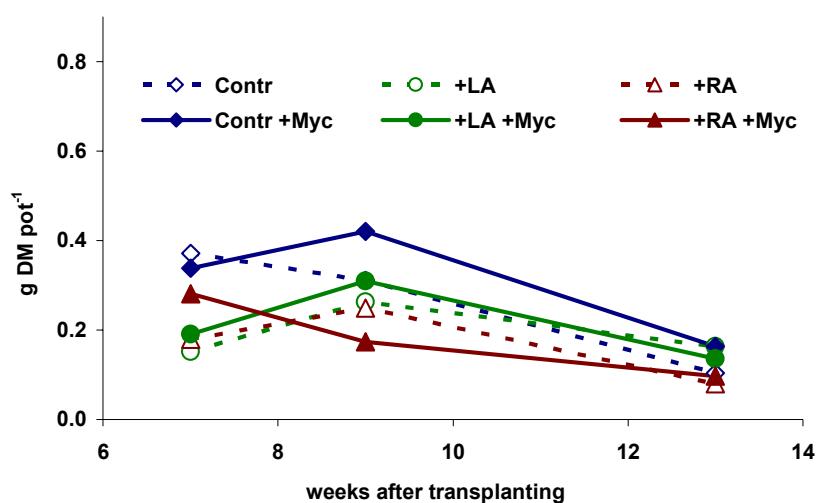


Fig. 3-14 – Root biomass of wheat grown in soil amended with maize leaves (+LA, green lines) or maize roots (+RA, red lines) or non amended soil (Contr, blue lines) and infected with *G. mosseae* (+Myc, continuous lines with closed symbols) or not (dashed lines with open symbols) at 7, 9 and 13 weeks after transplanting.

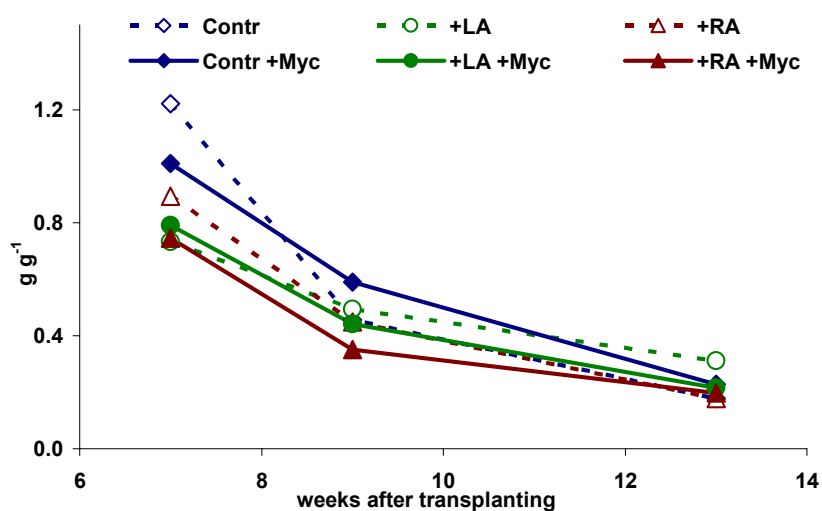


Fig. 3-15 – Root:Epigeic biomass ratio of wheat grown in soil amended with maize leaves (+LA, green lines) or maize roots (+RA, red lines) or non amended soil (Contr, blue lines) and infected with *G. mosseae* (+Myc, continuous lines with closed symbols) or not (dashed lines with open symbols) at 7, 9 and 13 weeks after transplanting.

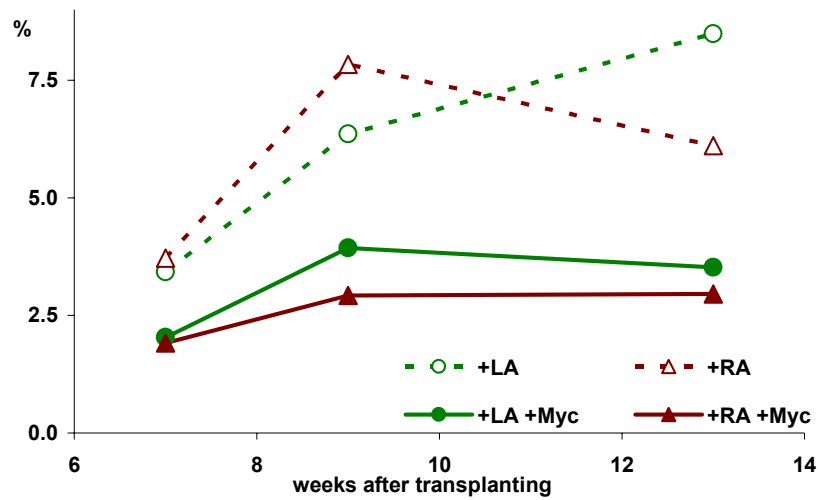


Fig. 3-16 –  $N$  recovery fraction ( $N_{REC}$ ) of wheat grown in soil amended with maize leaves (+LA, green lines) or maize roots (+RA, red lines) and infected with *G. mosseae* (+Myc, continuous lines with closed symbols) or not (dashed lines with open symbols) at 7, 9 and 13 weeks after transplanting.

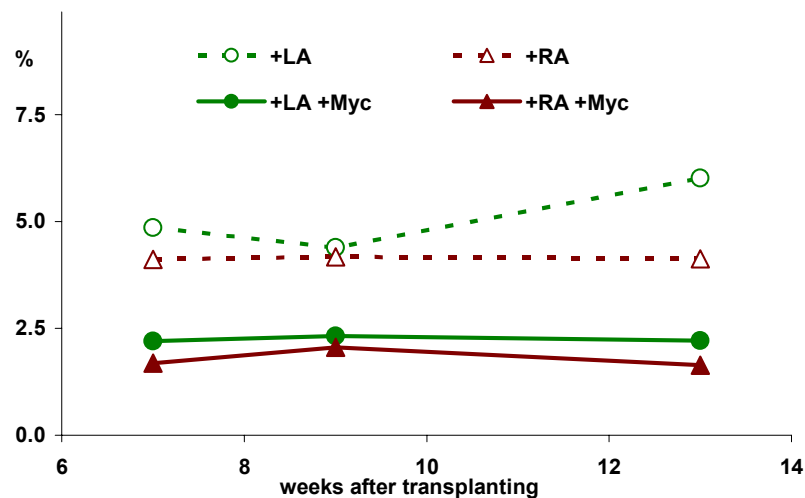


Fig. 3-17 – Percentage of plant  $N$  deriving from OM in wheat grown in soil amended with maize leaves (+LA, green lines) or maize roots (+RA, red lines) and infected with *G. mosseae* (+Myc, continuous lines with closed symbols) or not (dashed lines with open symbols) at 7, 9 and 13 weeks after transplanting.

Significances of data from soil enzymatic activity measurement in both planted and unplanted pots are given in Tab. 3-5. In unplanted soil, addition of maize leaves resulted in a higher enzymatic activity in comparison with no OM addition (Fig. 3-18), but its effects on casein hydrolyzing activity were observed in the first sampling date, only. Addition of maize roots didn't affect casein hydrolyzing and deamination activities in unplanted soil, but enhanced dehydrogenase activity in any of the sampling dates.

In planted soil, on average, all enzymatic activities were higher in leaves amended than non amended soil. Addition of maize roots to soil lowered urease and caseinase activities in comparison with non amended soil. The effects of AM symbiosis on soil enzymatic activity varied with OM treatment (Tab. 3-5 and Fig. 3-19). In particular, AM improved dehydrogenase activity at the first sampling in soil amended with maize root, only, and at the second sampling date in soil amended with both maize leaves and root. At the third sampling, soil dehydrogenase activity was higher in all AM than non AM treatments. Caseinase activity was improved at the first but depressed at the third sampling by AM symbiosis, while no difference among treatments were found at the second sampling. This effect is particularly evident in soil amended with maize leaves. AM symbiosis significantly improved BAA-protease in all samplings.

To compare DGGE profiles (Fig. 3-20), Nei & Li's indices were determined and UPGMA was used to create a dendrogram describing pattern similarities (Fig. 3-20a). The cophenetic coefficient calculated between the correlation matrix and the cluster tree was 0.78, indicating that the cluster tree was fit to explicate the difference or similarities between treatments. The dendrogram generated showed that bacterial profiles from non mycorrhizal wheat rhizosphere clustered together with a homology coefficient of about 66% (Fig. 3-21a, red coloured cluster), while bacterial profiles from AM wheat mycorrhizosphere clustered together with a homology coefficient of about 62% (Fig. 3-21a, blue coloured cluster). Bacterial profile by non cultivated non amended pots clustered with profiles from non mycorrhizal rhizosphere, while bacterial profiles from unplanted soil with leaves-amendant clustered with profiles from mycorrhizal rhizospheres. Bacterial profile from unplanted soil amended with maize roots and from the original bacterial inoculum clustered alone.

The structural diversity of the microbial communities was also examined by the Shannon ( $H'$ ) and Simpson ( $D_2$ ) diversity indexes (Tab. 3-6). Higher  $H'$  and  $D_2$  were found for bacterial communities of AM than non AM rhizosphere and for bacterial communities of non amended than leaves amended soils. Bacterial communities from root amended soils showed a higher degree of structural similarity to non amended soils than the ones from leaves amended soils. Tab. 3-6 also shows the  $S_{RC}$  values obtained from the comparison of soils from all treatments and from the original bacterial inoculum. No significant dissimilarities ( $S_{RC} < 0.05$ ) were found. A similarity greater than that expected by chance ( $S_{RC} > 0.95$ ) was observed in many cases, above all between amended treatments. Raup & Crick probability-based indices of similarity showed that AM treatment determined stronger change in soil bacterial communities when no OM was applied in comparison to those of OM added soils (Tab. 3-6). Fig. 3-20b shows the dendrogram generated by the Raup and Crick cluster analyses. The mantel test  $Z$  between matrices based on and DGGE banding pattern (UPGMA)



and enzymatic activities distances was 0.22, meaning little correlation between bacterial community structure and soil enzymatic activity.

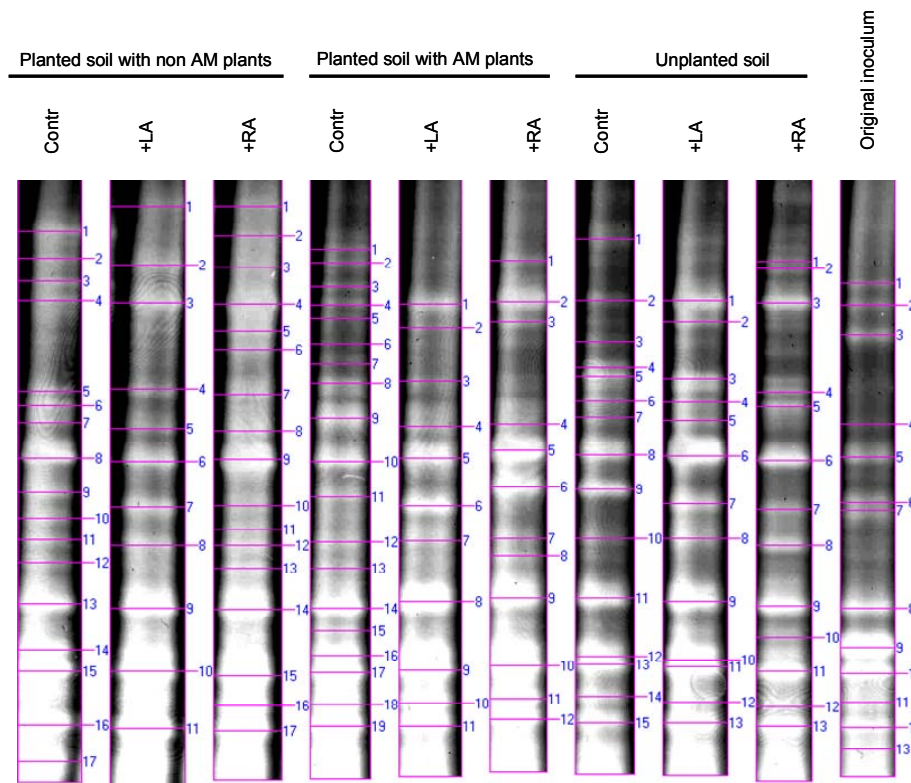


Fig. 3-18 – DGGE profiles from bacterial communities of soils from the applied treatments. Contr, +LA, and +RA for non amended, amended with maize leaves and amended with maize roots, respectively; Original Inoculum for bacterial community from the applied inoculum of microorganisms.

*Tab. 3-5 – Significances of treatments (OM, Myc, OM\*Myc for organic matter addition, AM inoculation treatments and interaction, respectively) at 7, 9 and 13 weeks after transplanting on enzymatic activity of soil from planted and unplanted pots. ns, non significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .*

Significance at different sampling moments											

Fig. 3-19 – (a) Dehydrogenase; (b) Casein hydrolyzing protease; (c) Urease; (d) and BAA- hydrolyzing protease in unplanted soil amended with maize leaves (+LA, dashed green lines with open circles) or maize roots (+RA, dashed red lines with open triangles) or not amended (dashed blue lines with open diamonds) at 7, 9 and 13 weeks. Different unit scales were used in urease and BAA- hydrolyzing protease graphs.

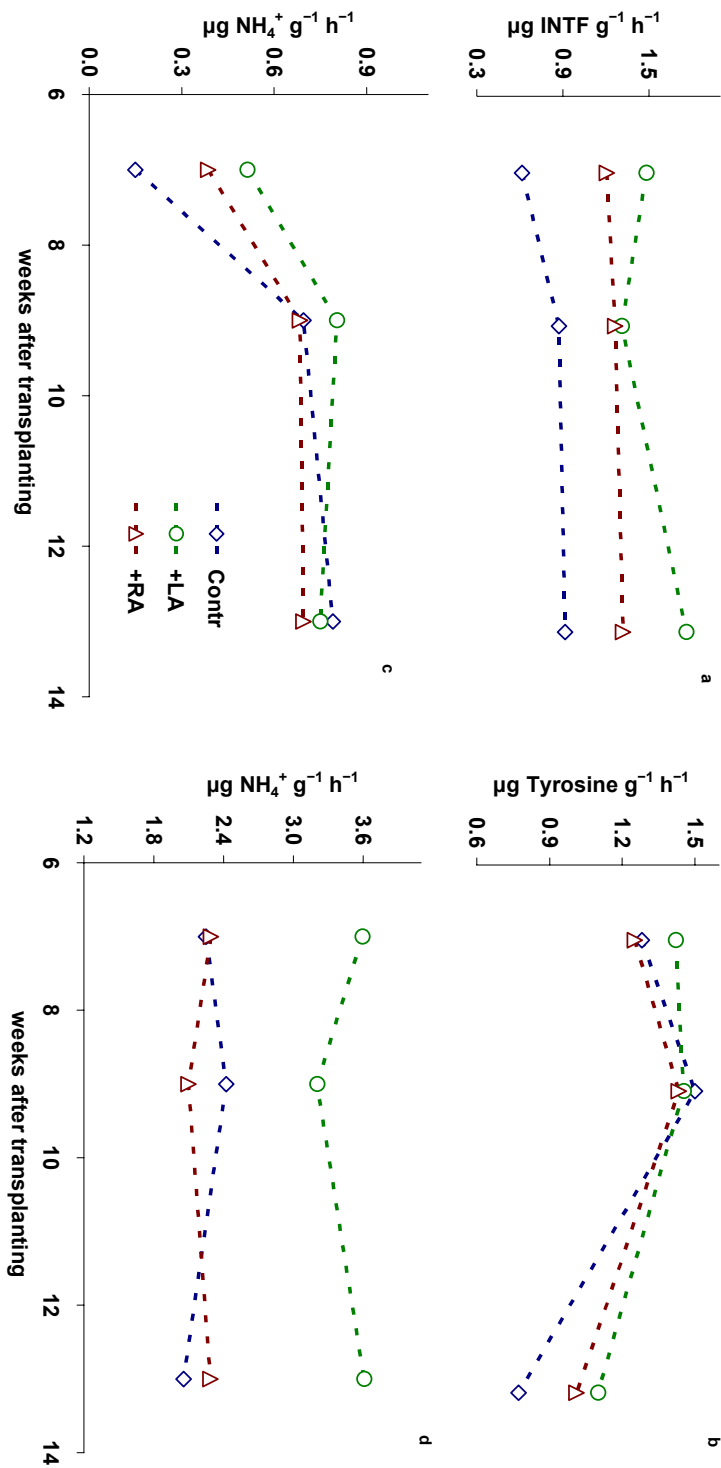
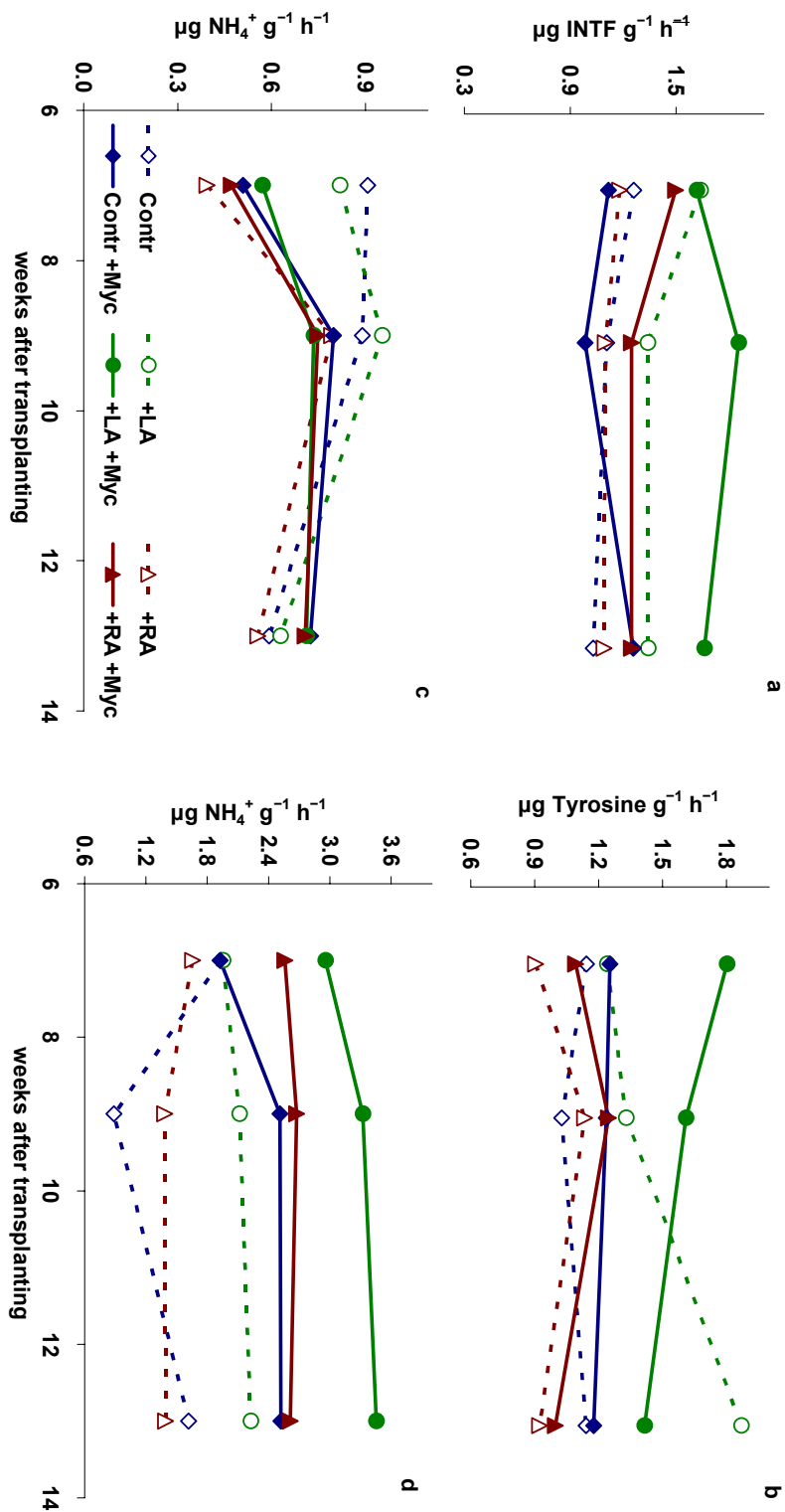


Fig. 3-20 – (a) Dehydrogenase; (b) Casein hydrolyzing protease; (c) Urease; (d) and BAA- hydrolyzing protease in planted soil amended with maize leaves (+LA, green lines with circles) or maize roots (+RA, red lines with triangles) or not amended (blue lines with diamonds) in which wheat plants were grown in symbiosis with the arbuscular mycorrhizal fungus *G. mosseae* (+Myc, continuous lines with closed symbols) or not (dashed lines with open symbols) at 7, 9 and 13 weeks after transplanting. Different unit scales were used in urease and BAA- hydrolyzing protease graphs.



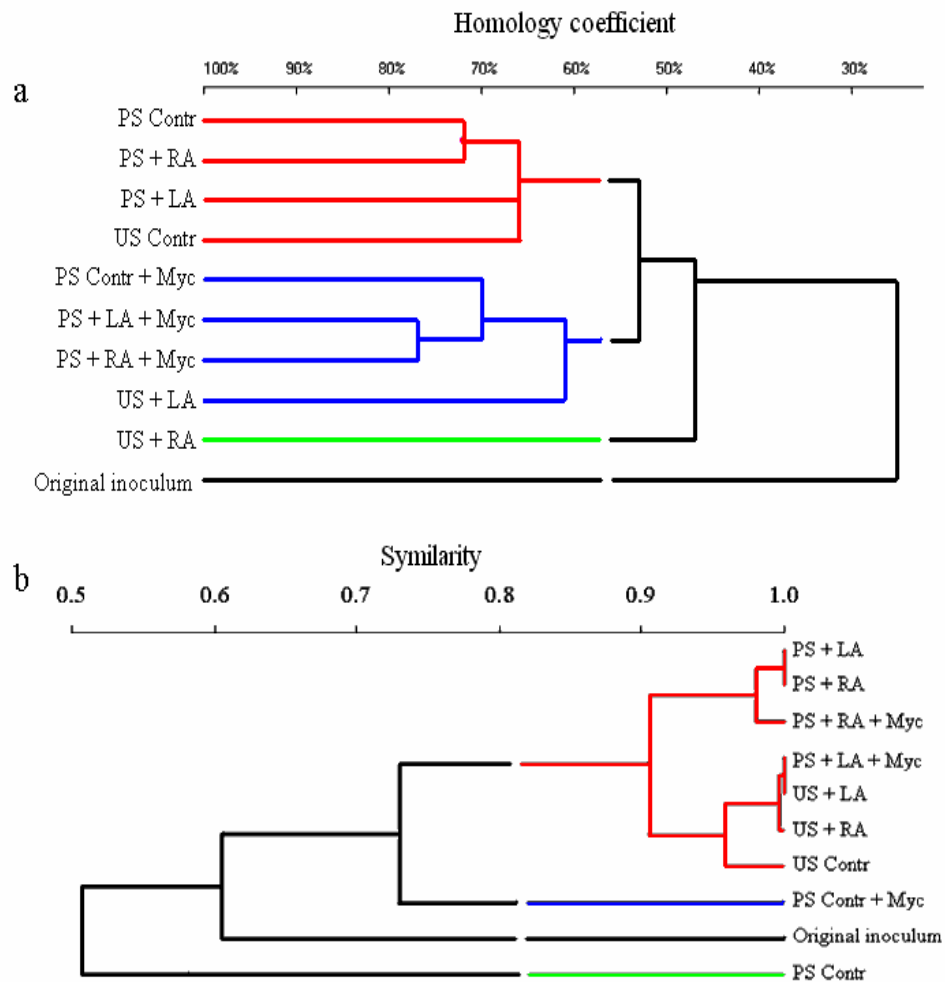


Fig. 3-21 – (a) Nei and Li's similarity coefficients (UPGMA dendrogram, tolerance 1.0%) and (b) Raup and Crick probability-based index of similarity cluster analyses for DGGE profiles from bacterial community structure of soils from the applied treatments. Each cluster with a different colour. PS, and US for planted and unplanted soil, respectively; Contr, +LA, and +RA for non amended, amended with maize leaves and amended with maize roots, respectively; +Myc for soil in which wheat was cultivated in association with AM fungus *G. mosseae*; Original Inoculum for bacterial community from the applied inoculum of microorganisms.

*Tab. 3-6 – Species richness, evenness and abundance, Shannon indices of general diversity and equitability, Simpson index of dominance of the bacterial community of soils from the applied treatments and Raup and Crick probability-based index of similarity between treatments.*

	Planted soil with non AM plants			Planted soil with non AM plants			Unplanted soil			Bacterial inoculum
	Control	Leaves amended	Root amended	Control	Leaves amended	Root amended	Control	Leaves amended	Root amended	
Species richness	S	17	11	17	19	11	12	15	13	13
Species evenness	$E=n/n_{\max}$	0.895	0.579	0.895	1.000	0.579	0.632	0.789	0.684	0.684
Species abundance	$A=n/N_{\text{tot}}$	0.472	0.306	0.472	0.528	0.306	0.333	0.417	0.361	0.361
Shannon index of general diversity	$H'$	2.17	1.61	2.21	2.87	1.60	1.99	2.63	2.06	2.13
Shannon's equitability	$E_H=H'/\ln S$	0.77	0.67	0.78	0.97	0.67	0.80	0.97	0.80	0.86
Simpson index of dominance (reported as reciprocal)	$1/D \text{ or } D_2$	16.20	10.30	16.11	16.69	10.52	11.79	14.34	12.89	11.41
Raup and Crick's probability-based index of similarity										
Planted soil with non AM plants	Control	0.72	<b>1</b>		<b>1</b>		<b>1</b>		<b>1</b>	
	Leaves amended	0.38								
	Root amended	0.38								
Planted soil with non AM plants	Control	0.11	0.79	0.58	0.81	0.93	0.93	0.92	0.83	0.67
	Leaves amended	0.68	<b>0.99</b>	<b>0.97</b>	<b>0.97</b>	<b>0.96</b>	<b>0.99</b>	<b>0.95</b>	<b>0.92</b>	<b>1</b>
	Root amended	0.79	<b>0.97</b>	<b>0.99</b>	<b>0.97</b>	<b>0.96</b>	<b>0.99</b>	<b>0.95</b>	<b>0.92</b>	<b>1</b>
Unplanted soil	Control	0.61	0.84	0.60	0.85	0.93	0.93	0.92	0.83	0.67
	Leaves amended	0.64	<b>0.96</b>	0.87	0.48	<b>1</b>	<b>0.99</b>	<b>0.92</b>	<b>1</b>	<b>1</b>
	Root amended	0.49	<b>0.99</b>	0.80	0.64	<b>1</b>	<b>0.95</b>	<b>0.92</b>	<b>1</b>	<b>1</b>
bacterial inoculum		0.15	0.85	0.39	0.17	0.93	0.71	0.30	0.83	0.67

### 3.2.2 DISCUSSION

Percentage of root AM colonization of wheat increased until the second sampling (9 week after transplanting) and remained constant until the third one. In spring wheat, Zhu & Smith (2001) observed that AM colonization reached its highest values at the seventh week of growth. On average, root AM infection was similar to that observed by other authors in pot studies (Li *et al.*, 2005 and 2006), but lower than that observed under field condition by Al Karaki *et al.* (2004). Probably, as shown by several authors (Bååth & Hayman, 1984; Koide, 1991; Allsopp & Stock, 1992), in pot the fine root system of wheat is able to completely explore the available soil volume and in such conditions the cost:benefit ratio of AM symbiosis for nutrient uptake is reduced, thereby plants reduce the amount of C fed to the AM fungus and root AM infection decreases. The highest values of ALP and SDH activities were observed at 9 WAT (2<sup>nd</sup> sampling), in accordance with Zhu *et al.* (2001). ALP is related to the rate of P transfer from AM fungi to plants and can be used as marker for analyzing the AM symbiotic efficiency (Tisserant *et al.*, 1993). Since wheat shows high P requirements during the first 7-9 weeks after emergence (Römer & Schilling 1986) and such requirements are not easy to satisfy by root uptake, it's expectable that during this phase, plants show a certain dependency on AM symbiosis for P absorption (Zhu & Smith, 2001).

Addition of OM exerted a depressive effect on plant growth and N uptake, in accordance with other studies (Seligman *et al.*, 1986; Hodge *et al.*, 1998). Considering that addition of OM increased the soil microbial activity, as data from dehydrogenase activity suggest, and that soil microorganisms compete better than plants for nutrients on a short timescale (Jackson *et al.*, 1989; Kaye & Hart, 1997), it's possible that this depressive effect was due to a higher sequestration of available inorganic N by microorganisms in OM than non amended soil. On the other hand, it has been shown that bacteria decreased N availability for plant uptake when C:N ratio of the added organic residues is higher than 12.5 (Killham, 1994; Hodge *et al.*, 2000a).

On average, plants inoculated with *G. mosseae* (BEG 12) yielded 15,6% more biomass relative to the non-mycorrhizal plants. Other researches have shown that growth response of wheat to AM infection is variable (Al-Karaki & Al-Omoush, 2002; Li *et al.*, 2005 and 2006) in relation to many conditions such as genotypic differences in root morphology, nutrient uptake and symbiotic efficiencies or to different growth conditions, in particular, nutrient availability. Several researches highlighted that AM symbiosis improve plant growth and nutrient uptake especially when plants are grown under nutrient limiting conditions (Azcón & Ocampo, 1981; Vierheilig & Ocampo, 1991; Karagiannidis & Hadjisavva-Zinoviadi, 1998), such as in the present study.

In accordance with other studies (Tobar *et al.*, 1994; Azcón *et al.*, 2001), a higher N uptake was observed in AM than non AM plants. It has been shown that by means of the AM symbiosis, plants can better scavenge the soil volume (Miyasaka & Habte, 2001) so enhancing the possibility to absorb the available N. This effect is particularly important when AM extraradical hyphae explore soil volumes different of those explored by the plant root system (Hodge *et al.*, 2001), such as in field conditions; whereas in pot, the wheat root system *per se* is able to explore the entire soil volume and it implies a reduction of the advantage of AM symbiosis for nutrient uptake. Therefore, as suggested by Hodge *et al.* (2000a), AM symbiosis could have improved N

uptake in host plants by being more effective than the sole roots to compete for inorganic nitrogen with soil microorganisms. An indirect confirmation of this mechanism can be found in the higher proteolytic activity observed in the non AM than AM rhizosphere. Infact, considering that soil proteases are inducible enzymes which are progressively released when organic:inorganic soil N ratio increases (Gill & Modi, 1981; Chrost, 1991), it's conceivable that the higher proteolytic activity in AM than non AM treatment depended by a mycorrhizal depletion of inorganic nitrogen, given that, to my knowledge, there's no evidence regarding the secretion of proteases by AM fungi.

In non AM treatments, at 13 weeks after transplanting, the percentage recovery of  $^{15}\text{N}$  from OM ( $\%N_{\text{REC}}$ ) was 8.49% and 6.11% when maize leaves or maize roots, respectively, were added to soil. These differences can be due to the different C:N ratio of the added OM (higher in maize roots than leaves). Indeed, the rate of mineralization of the OM strictly depends on the C:N ratio of the substrate being decomposed: the lower its C:N ratio, the higher its mineralization rate. In a 39 days long trial, Hodge *et al.* (1998) observed in different grasses values ranging from 3.2% to 5.0% of the  $\%N_{\text{REC}}$  from OM when *L. perenne* shoots with a 31:1 C:N ratio were added to soil, whereas in a 49 days long trial, the same authors (Hodge *et al.*, 2000c) found a  $\%N_{\text{REC}}$  value of 11% when *L. perenne* shoots with a 21:1 C:N ratio were added to soil.

The root infection with AM fungi strongly reduced the  $\%N_{\text{REC}}$  of the N applied with OM (-50% on average in comparison to non AM control); at the same time, the soil proteolytic activity was higher in AM than non AM treatments, which, in theory, should imply a higher rate of N mineralization and therefore a higher availability of N from organic sources; so it should have improved the  $\%N_{\text{REC}}$  from OM in AM than non AM treatments. Also Hodge *et al.* (2000b) showed that the AM symbiosis accelerates decomposition of fresh organic matter, without promoting an increase of  $\%N_{\text{REC}}$  from organic matter. In order to explain the decrease of  $\%N_{\text{REC}}$  observed in AM than non AM treatments, I hypothesize that microorganisms and AM plants differentially competed for the different sources of N (from soil or OM): AM plants better competed for soil mineral N, whereas bacteria better utilized N from added OM. Other research highlighted that, on a short timescale, microorganisms compete better for N from organic sources (Nadelhoffer *et al.*, 1985; Kaye & Hart, 1997; Hodge *et al.*, 2000a) and so, the reduction of the  $\%N_{\text{REC}}$  in AM than non AM treatments could be due to a temporal sequestration of N from organic sources by bacteria. An indirect confirmation of this hypothesis came from Hodge (2003), who found, at harvest, a higher percentage of N mineralized from the applied organic matter in soil of AM than non AM treatments.

Addition of OM to soil consisted in a strong change in the enzymatic profile and bacterial community structure of both planted and unplanted soil with a overall increase in both proteolytic and dehydrogenase activity of soil microorganisms. It has been shown, under controlled conditions, that the addition of fresh organic matter to soil trigger into activity many dormant r-strategist microorganisms (De Nobili *et al.*, 2001) and it may exert a priming effect on mineralization activity of stable soil organic matter (Fontaine *et al.*, 2003). In the present experiment, a lower protease activity was observed in soil amended with maize roots than maize leaves. It is likely that these differences somehow depended on the chemical-bromatologic composition of the material being decomposed (Bonmatí *et al.*, 2009).



The addition of organic matter decreased  $H'$  and  $D_2$  from the DGGE banding patterns, suggesting a decrease in bacterial diversity. It is possible that the addition of OM increased the competitiveness of the r-strategist bacteria both for preexistent and added nutrients (Andrews & Harris, 1986; Fontaine *et al.*, 2003), with a subsequent decrease of the abundance of the K-strategist bacteria. This decrease was marked so much so the abundance of K-strategist bacteria was under the detection limit of DGGE protocol. A similar result was obtained by Dilly *et al.* (2004).

Plant inoculation with AM fungi determined an increase in  $H'$  and  $D_2$  from the DGGE banding patterns, suggesting that bacterial diversity increased in AM treatments than non AM ones. Marschner *et al.* (2001) found a lower bacterial species abundance in rhizosphere of plants inoculated with *G. mosseae* than non mycorrhizal plants, whereas Amora-Lazcano *et al.* (1998) observed an increase in the number of ammonia oxidizers and a decrease in the numbers of ammonifying and denitrifying bacteria due to plant inoculation with AM fungi. Moreover, Mansfeld-Giese *et al.* (2002) observed a selective mycorrhizal influence on bacterial strains. Indeed, AM fungal infection alters root exudation qualitatively and quantitatively (Graham *et al.*, 1981; Dixon *et al.*, 1989; Po & Cumming, 1997; Marschner *et al.*, 1997), which preferentially stimulate ones while depressing other bacterial functional groups, so that it's conceivable that AM influence on bacterial community is mediated by the AM effects on host plant and also by a selective AM influence on rhizosphere populations (Andrade *et al.*, 1998).

The average increase of the enzymatic activity due to the addition of maize leaves to soil was associated to a decreased abundance of the bacterial species, as suggested by the decrease of  $H'$  and  $D_2$  indices. On the other side, the higher enzymatic activity (especially BAA- and casein- hydrolyzing activities) due to plant AM inoculation wasn't related to qualitative change in the microbial community. Similar results were achieved by Hodge *et al.* (2001) who found an increase of mineralization activity due to plant AM colonization, but no qualitative changes in microbial community described by phospholipids fatty acid profiling. The latter authors hypothesized that variations in mineralization activity of organic matter were probably a direct result of the presence of the AM fungal hyphae, suggesting that AM fungi could have saprotrophic capabilities, although the mechanisms involved are still unknown.

## **4 CONCLUSIONS AND FUTURE PROSPECTS**

The present research aimed to obtain information about the contribution of the AM symbiosis in alleviating the effect of abiotic stresses to Mediterranean crops and to test if AM symbiosis affects symbiotic N<sub>2</sub> fixation and organic N mineralization activities and finally plant N uptake.

In the field trial, the AM symbiosis improved both plant growth and symbiotic N<sub>2</sub> fixation of berseem grown under drought-stressed, but not under well-watered conditions and it suggests that the AM symbiosis can contribute to crop production in Mediterranean environments where spring rainfall is scarce, which stresses plants and limits crop productivity. Therefore, in such environments, when choosing a management practice (such as tillage, fertilization, etc.) to be applied, it should also be taken into account the aspects concerning the preservation of the natural AM population. Moreover, a valuable agronomical practice could be the inoculation with effective AM fungal strains in order to improve both the natural AM population and the efficiency of the AM symbioses. However, the applicability of this solution needs further studies in order to identify effective combinations of AM strains and plant genotypes and to investigate on the competition between natural and introduced AM fungi in soil.

In the pot trial, the AM symbiosis improved total N uptake in plants and soil N mineralization rates, suggesting that the AM activity is important in improving both plant growth and soil quality. However, AM symbiosis decreased plant N recovery from organic sources, probably due to a different ability between microorganisms and AM plants in utilizing the different sources of N (from soil or OM). The increase of soil enzymatic activity in the AM treatment comparing to non AM counterpart wasn't related to qualitative changes in the microbial community and it suggests a direct effect of the AM fungi on the decomposition rate of OM, possibly through an association with particular functional group of soil microorganisms. This latter aspects need further research because an advancement of knowledge about the role and impact of AM fungi on the decomposition process could surely have practical implication on crop production in Mediterranean environments.

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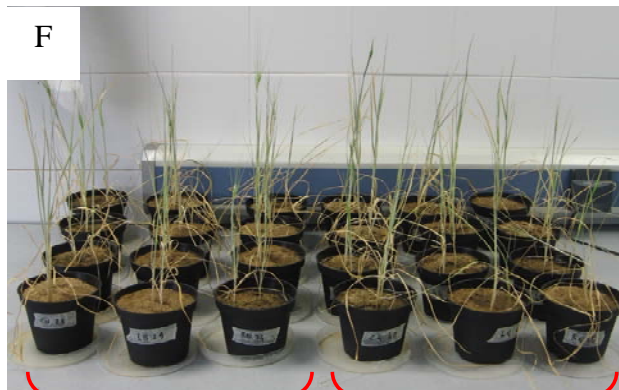
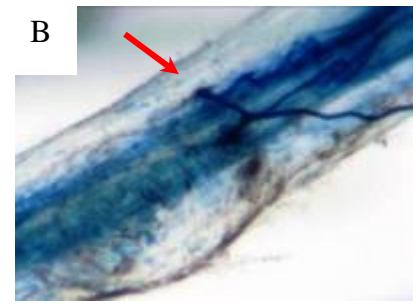
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Non mycorrhizal plants

Mycorrhizal plants

Some pictures of the experiments:

A: experimental field.

B: wheat root after staining with trypan blue (red arrow indicates a mycorrhizal infection point).

C: berseem plot after cut.

D: berseem sample area after removal of roots.

E: sample areas in a berseem plot.

F: not infected and mycorrhizal wheat plants (the common “little plant/big plant” effect was probably impaired by the nitrogen lack).